TAXONOMY AND BIOCONTROL OF *DIAPORTHE SOJAE* AND SCREENING FOR RESISTANCE TO PHOMOPSIS SEED DECAY CAUSED BY AN ATYPICAL *DIAPORTHE SOJAE* ISOLATE USING VARIOUS ASSAYS

BY

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THESIS

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Abstract

Phomopsis longicolla, a seedborne fungal pathogen of soybean, has been known to be the main cause of Phomopsis seed decay (PSD), which is a major cause of poor seed quality in the soybean growing regions of the United States but especially in the Mid-South. This species has been morphologically and pathologically distinguished from *Diaporthe phaseolorum* var. *sojae*; however, most of the characteristics thought to distinguish the two species have been reported and found presently to overlap. Because these two fungi cannot be distinguished morphologically, pathogenically, or phylogenetically, it is likely that they are the same species and the name *Diaporthe sojae* should be assigned to them. Breeding for resistance to PSD is often a time-consuming endeavor as soybeans need to be grown to maturity before plating seed on agar plates can be done to assay for PSD resistance. Thus, a mature seed inoculation assay, an immature stem assay, and a detached leaf assay were conducted to see if soybean genotypes resistant to PSD would prove to be similarly resistant in any of these assays. Results showed that the mature seed inoculation assay and the detached leaf assay cannot be used to screen for PSD resistance while conditions more favorable to colonization by *D. sojae* would help verify the utility of the immature stem assay. In addition to the investigation of alternative PSD resistance screening assays, the standard screening assay for PSD resistance that is usually conducted in the field was attempted in the greenhouse. The results from the greenhouse assays reinforced the importance of controlling relative humidity and temperature in obtaining informative PSD incidences, incidences that are statistically different between the susceptible and resistant controls. Planting PSD-resistant cultivars is likely the most effective method of controlling PSD, but such cultivars have yet to be developed. Other management strategies exist, such as later planting to avoid hot and humidity weather during the seed development and maturity stages (R5-R8) and fungicide use, but an effective biological control of PSD has yet to be developed.

Therefore, *Acremonium strictum*, a fungus that was found to parasitize an isolate of *D. sojae*, was tested in vivo, ex vivo, and in vitro for its biocontrol capabilities. The findings from the series of experiments showed that *A. strictum* acts as a protectant rather than a curative biocontrol and that it does inhibit mycelial growth in vitro, albeit with a delay. Further testing would be required to quantify the protectant effects of *A. strictum* on PSD incidence in the greenhouse or in the field.

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Chapter 1: Introduction and Literature Review

Introduction

Soybean (*Glycine max* (L.) Merr.) has become the agronomic crop to grow in the Midwest along with corn ever since World War II increased domestic demand for edible fats and oils (North Carolina Soybean Producers Association, Inc.). Additionally, the rapid increase in domestic meat consumption required a cheap source of protein for livestock, and soybean meal was the preferred option for livestock feed. With the spread of soybean monoculture came inevitable disease pressure on the emerging crop. It has been estimated that over 484 million bushels of soybean were lost to disease in 2007 (Wrather and Koenning 2009). In addition to crop yield loss, pathogens, such as *Phomopsis longicolla* Hobbs, *Cercospora kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner, *Fusarium* spp. Link, and soybean mosaic virus, can cause soybean seed malformation and/or discoloration (Sinclair 1995). The value of such seed is discounted at the grain elevator due to the assignment of lower grades (Federal Grain Inspection Service 2004). Therefore, by reducing the likelihood that these pathogens will infect soybean and cause deleterious symptoms on the seed, we can increase the quality of soybean seed.

The *Diaporthe*/*Phomopsis* complex (DPC) contains soybean fungal pathogens that have caused estimated yield losses of over 45 million bushels in 2009, with 23 million bushels attributable to Phomopsis seed decay (PSD) (Koenning and Wrather 2012). However, Mengistu et al. (2009) found no consistent correlation between yield and PSD and Bisht and Sinclair (1985) did not find a significant yield difference between plots inoculated with *Diaporthe phaseolorum* var. *sojae*, which had higher levels of PSD than the control plots, and control plots. Nevertheless, moldy seed, which is one symptom of PSD, can discount soybeans at the grain elevator (Federal Grain Inspection Service 2004). Additionally, PSD causes a reduction in the germination, emergence,

and vigor of seed (Begum et al. 2008; Bisht and Sinclair 1985; Chamberlain and Gray 1974; Ellis et al. 1974; França Neto et al. 1985; Franca Neto and West 1989; Gillen et al. 2012; Grau and Oplinger 1981; Hepperly and Sinclair 1980b; Kmetz et al. 1978; Li et al. 2011; Mayhew and Caviness 1994; McGee et al. 1980; Mengistu and Sinclair 1979; Paschal and Ellis 1978; Roy et al. 1997; Smith et al. 2008a; Spilker et al. 1981; TeKrony et al. 1984; TeKrony et al. 1987; TeKrony et al. 1996; Wallen 1960; Wallen and Cuddy 1960; Wilcox et al. 1974; Wilcox et al. 1985; Wilcox and Abney 1971); therefore, this disease is a problem for seed companies trying to obtain high-quality seed to sell to farmers.

PSD has deleterious effects on soybean and those who consume them. Soy flour produced from seed obtained from seed lots with symptomatic seed may have a moderately pronounced unpleasant odor, which causes products produced from such flour to be unpalatable (Hepperly and Sinclair 1978). Additionally, oil produced from seed obtained from seed lots with symptomatic seed may have a lower visual quality, a rancid odor, and higher peroxide values, indicating oil deterioration. Furthermore, seed obtained from seed lots with symptomatic seed had significantly greater oil content and protein content. However, Fábrega et al. (2000) found that protein content significantly decreased in seed that had a level of PSD while oil and free fatty acid content significantly increased. PSD can also produce seed that have altered fatty acid compositions; specifically, PSD is significantly correlated with decreased palmitic and oleic acid composition and increased linoleic and linolenic acid composition (Wrather et al. 2003). However, isogenic seed manipulated environmentally to have significantly higher oleic acid and an oleic:linoleic acid ratio and significantly lower linoleic and linolenic acid did not show a decrease in the colonization potential of *D. phaseolorum* on the cotyledons of such seed (Xue et al. 2008). The increase in linolenic acid is likely to be the cause of the off odor Hepperly and

Sinclair (1978) observed (Wrather et al. 2003). Decreased levels of palmitic acid are good for human health as palmitic acid increases LDL levels, which are associated with heart disease, whereas decreased levels of oleic acid are unfavorable as oleic acid is a beneficial monosaturated fatty acid. On the other hand, Fábrega et al. (2000) did not find a significant difference in fatty acid composition between seed that had a high level of PSD and seed that had no PSD. Also, they found no difference in soluble protein composition.

When one-day-old chicks were intubated with a high dose of a filtrate of *P. longicolla*, the primary fungus causing PSD, grown on soybeans some of them died (Kung et al. 1976; Kung et al. 1979). Weight loss and reduced consumption of feed in the chicks was also observed (Kung et al. 1979). Additionally, they experienced reduced plasma glucose, reduced histologically observed glycogen and enzymatically determined glycogen concentrations, necrosis, vacuolization, and hemorrhage in the liver, and increased glucose-6 phosphatase and glucose-6-phosphate dehydrogenase activity (Kung et al. 1976; Kung et al. 1977; Kung et al. 1979). Increased glucose-6 phosphatase in gluconeogenesis is one way that glucose levels in the blood can be restored, the other being glycogenolysis (Kung et al. 1979). Increased glucose-6-phosphate dehydrogenase is thought to be the liver's way of speeding up oxidation of the toxin from *P. longicolla* with the aid of NADPH, the means by which the liver is supplied with pentose sugars for nucleic acid production in the repair of cells damaged by the toxin, or both (Kung et al. 1976; Kung et al. 1979). When one-day-old chicks were intubated with a filtrate of *P. longicolla* grown on soybeans over a period of six weeks they gained less weight, had larger livers than the control, and had decreased plasma albumin (Kung et al. 1977). Decreased plasma albumin might be due to reduced protein synthesis caused by the toxin. Reduction of lipoprotein production is likely the cause of fat accumulation in the liver due to the inability of triglycerides

to leave the liver. Between two and three weeks of intubation, mycotoxicosis seems to occur in chicks, with symptoms such as increased fat content, biliary hyperplasia, Kupffer cell hypertrophy and hyperplasia, and endothelial hyperplasia in the liver. Additionally, *P. longicolla* grown on wheat is highly to moderately toxic when intubated into six month old castrated male sheep causing extreme necrosis of the epithelium in the reticulum, rumen, and omasum (Allen et al. 1992).

Cytochalasins have also been found to be produced by members of the DPC. Cytochalasins are a class of fungal metabolites that have been found to cause a number of deleterious effects on mammalian cells, including inhibition of cell movement and cytoplasmic cleavage and partial or complete nuclear extrusion (Carter 1967). Epoxycytochalasin H and epoxydeacetylcytochalasin H from *D. phaseolorum var. sojae* caused death in day-old chicks intubated with either purified [11]cytochalasin and inhibited growth of wheat coleoptiles (Cole et al. 1982). Putative cytochalasin H and J have been recovered from *P. longicolla* but not from other *Diaporthe* or *Phomopsis* spp. (Allen et al. 1992).

Literature Review

The *Diaporthe*/*Phomopsis* complex diseases

The *Diaporthe*/*Phomopsis* complex (DPC) members belong to the order Diaporthales and family Diaporthaceae and cause a number of diseases on soybean: Phomopsis seed decay (PSD), caused primarily by *P. longicolla*; pod and stem blight, caused primarily by *D. phaseolorum* var. *sojae*; northern stem canker and top dieback, caused primarily by *D. phaseolorum* var. *caulivora*; and southern stem canker, caused primarily by *D. phaseolorum* var. *meridionalis* (Sinclair 1999; Rossman et al. 2007). The division between northern and southern stem canker is relatively

recent and both diseases were formerly simply referred to as stem canker. These pathogen-disease complexes are not absolute: *P. longicolla* and *D. phaseolorum* var. *sojae* have been shown to cause stem cankers, and *P. longicolla* has been shown to cause pod and stem blight (Cui et al. 2009; Kmetz et al. 1979; Vidić et al. 1998). *P. longicolla*, *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* can all infect soybean seed, and the first three of these fungi have been shown to cause seed rotting and molding with *P. longicolla* being the most virulent pathogen among the three (Asante et al. 1998; Kmetz et al. 1974; Kmetz et al. 1979; Sun et al. 2012b). Although Jeffers et al. (1982) and Kmetz et al. (1978) in Ohio, TeKrony et al. (1983) in Kentucky, Anderson et al. (1985) in Ontario, Brown et al. (1987) in Missouri, Mengistu et al. (2010) in Mississippi, and Raeisi et al. (2011) in Malaysia found that *P. longicolla* is the most prevalent member of the DPC found in soybean seed, others have found *D. phaseolorum* var. *sojae* to be the most prevalent species in seed from Puerto Rico (Brown et al. 1987), Indiana (Baird et al. 2001; Ploper et al. 1992), and South Korea (Oh 1988). Xue et al. (2007), however, did not find a significant difference between the isolation of *P. longicolla*, *D. phaseolorum* var. *sojae*, or *D. phaseolorum* var. *caulivora* from seed in Ontario, although seed infection by DPC species was infrequent in their study.

The symptoms of PSD are discoloration, fissuring or splitting, flattening, shrinking, shriveling, wrinkling, and ellipsoid elongation of seed (Casady et al. 1992; Hepperly and Sinclair 1978; Jeffers et al. 1982; Lehman 1922; Lehman 1923). Wrinkling has been shown to be positively correlated to PSD (Smith et al. 2008a). White, grayish-white, or gray mycelia sometimes cover the seed, giving it a white to gray or a gray-green color (Crittenden et al. 1967; Hepperly and Sinclair 1978; Jeffers et al. 1982; Lehman 1922; Lehman 1923). A tan, brown, or charcoal gray appearance on the seed has also been reported (Casady et al. 1992). Additionally, the seed

texture can have brown and white stripes. When diseased seed are cut open, they have a greenish-brown or grayish-brown appearance rather than a yellow color (Crittenden et al. 1967). Seed coat etching has been shown to not be related to PSD (Thomison et al. 1989). PSD has also been shown to decrease seed weight, volume, density, roundness, sphericity, and surface area while increasing breakage susceptibility (Bisht and Sinclair 1985; Fábrega et al. 2000; Hepperly and Sinclair 1978; Lehman 1923; Mbuvi et al. 1989). However, some have found PSD to be positively correlated with seed weight and size while other have found PSD to not affect seed weight (Li et al. 2011; Manandhar et al. 1987a; Mengistu et al. 2009; Paschal and Ellis 1978; Smith et al. 2008a). PSD has been associated with suppressed germination, emergence, and vigor (Begum et al. 2008; Bisht and Sinclair 1985; Chamberlain and Gray 1974; Ellis et al. 1974; França Neto et al. 1985; Franca Neto and West 1989; Gillen et al. 2012; Grau and Oplinger 1981; Hepperly and Sinclair 1980b; Kmetz et al. 1978; Li et al. 2011; Mayhew and Caviness 1994; McGee et al. 1980; Mengistu and Sinclair 1979; Paschal and Ellis 1978; Roy et al. 1997; Smith et al. 2008a; Spilker et al. 1981; TeKrony et al. 1984; TeKrony et al. 1987; TeKrony et al. 1996; Wallen 1960; Wallen and Cuddy 1960; Wilcox et al. 1974; Wilcox et al. 1985; Wilcox and Abney 1971). Furthermore, Mengistu et al. (2009) found that seed germination was only negatively correlated with PSD in post-flower-irrigated and pre- and post-flower-irrigated environments but not in the non-irrigated environment. In contrast, in a multiyear, multilocational experiment that used soybean seed from a variety of sources, no single test of germination, vigor, or emergence significantly correlated to PSD in every year, seed, location, and seed source combination, but the official towel test's measurement of total normal seedlings provided the simplest way of obtaining a relatively good indicator of PSD (Kulik and Schoen 1981). However, this test may not give an accurate representation of germination capacity of

seed with PSD because if only the seed coats of such seed are infected and not the embryos, the seed may germinate to a high degree due to the infected seed coat dehiscing after germination (Franca Neto and West 1989). It should be noted that poor-geminating seed infected with members of the DPC do not always exhibit visual PSD symptoms. Plump seeds free of discoloration have been found to be infected with DPC members and/or to have reduced germination rates (Anderson et al. 1985; Chamberlain and Gray 1974; Grau and Oplinger 1981; Lehman 1922; Peterson and Strelecki 1965; Wallen and Cuddy 1960). Therefore, pre-emergence damping-off, either with or without a visual sign or symptom on a seed, is another symptom of PSD.

Root and basal stem rot symptoms on seedlings germinating from *D. phaseolorum* var. *sojae*-infested soil include cotyledons with brown lesions that turn yellow and drop off prematurely, brownish-red lesions on the lower hypocotyl and upper taproot, basal girdling, and death of rootlets (Gerdemann 1954). Month-old plants also grew to be shorter than ones grown in non-infested soil. Slightly sunken lesions of variable size and shape containing pycnidia and bordered by a think black margin have been found on basal stems and have been found to be caused by *P. longicolla* (Vidić et al. 2013). Upper taproot infection by *P. longicolla* has been found in the field as early as the V1 stage (Fehr et al. 1971; Mengistu et al. 2009). Additionally, seedlings germinating from *D. phaseolorum*-infected seed were found to be stunted and to have short primary roots, brown lesions on the cotyledons and on the bases of hypocotyls, and deformed or decayed primary buds (Peterson and Strelecki 1965; Wallen and Cuddy 1960).

Pod and stem blight is characterized by the development of pycnidia on pods and stems, with the latter having the pycnidia arranged in linear rows (Lehman 1923). Northern stem canker starts off as a reddish-brown leaf scar that then develops into a slightly sunken canker several inches

long enveloping the stem (Athow and Caldwell 1954). Top dieback is characterized by the upper internodes of the plant becoming dark brown relative to the lower internodes of the plant (Hobbs et al. 1981). Southern stem canker begins as a lesion at the intersection nodes of the main stem and a lateral branch or petiole, or less often on a stem or petiole, that is dark in the center and has a light border, but as the disease progresses, the lesion becomes light in the center and dark at the border (Yorinori 1990). After maturity, however, the canker acquires the color of the plant, which makes detection of cankers difficult. As the canker grows up and down the main stem, the fungus eventually reaches the pith, which turns red-brown before plant maturity and turns light brown or purplish after maturity, and kills the plant with the possibility of stem breakage and lodging if planting was delayed. Pith symptoms are more pronounced near the intersection nodes but are still present beyond the lesions. The leaves experience interveinal chlorosis and necrosis and ultimately die and turn brown.

History of the *Diaporthe*/*Phomopsis* complex

The taxonomy of fungi in the DPC that cause PSD and other diseases on soybean has been complicated. Wolf and Lehman (1920) reported *Phoma* blight on pods and stems of soybean caused by what they thought was a *Phoma* sp. In a short disease note, Lehman (1922) later named the causal organism of pod and stem blight *Phomopsis sojae*. Pycnidia were observed on the stems, pods, and leaves, but there was no mention of the presence of perithecia. Seed in diseased pods were enveloped in white mycelia and shriveled (Lehman 1922). Following more extensive study of the causal organism of pod and stem blight, Lehman (1923) named it *Diaporthe sojae* because its anamorph characteristics differed from those of *Diaporthe phaseolorum*, the cause of pod blight on lima bean. Also, unlike *D. phaseolorum*, it had not been observed to produce perithecia in the field. In 1924, however, perithecia were found on

overwintering soybean stems in the field in North Carolina (Wolf and Lehman 1926). Although Lehman (1923) referred to the anamorph of *D. sojae* as the *Phomopsis* stage of *D. sojae* rather than *P. sojae*, an anonymous reviewer (1923) later linked *P. sojae* as the anamorph of *D. sojae*. *Phomopsis glycines*, a fungal species on soybean described by Petrak (Petrak and Sydow 1936), was found to be synonymous with *P. sojae* due to their overlapping morphological characteristics (Hobbs et al. 1985). Wehmeyer (1933), however, did not think that *D. sojae* was morphologically different enough from *D. phaseolorum* to warrant creating a new species name for it and believed *D. sojae* was a variety of *D. phaseolorum*, renaming it *Diaporthe phaseolorum* var. *sojae*. Furthermore, Wehmeyer (1933) also did not think that *Diaporthe batatatis*, the causal organism of dry rot in sweet potato (Harter and Field 1912), was morphologically different from *D. phaseolorum* either and renamed it *Diaporthe phaseolorum* var. *batatatis*. Welch and Gilman (1948) attributed the causal organism of stem canker to a perithecial strain of *D. phaseolorum* var. *batatatis*, although they did not directly work with a *D. phaseolorum* var. *batatatis* isolate. Upon investigating the pathogenicity and morphology of *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *batatatis*, and a fungus isolated from a stem canker infection on soybean, Athow and Caldwell (1954) found that their putative *D. phaseolorum* var. *batatatis* isolate did not cause stem canker and proposed that the fungal variety that causes stem canker in soybean be named *Diaporthe phaseolorum* var. *caulivora*. Kulik (1984) did not see a difference between *D. phaseolorum*, *D. phaseolorum* var. *sojae*, and *D. phaseolorum* var. *batatatis* and considered their correct anamorph and teleomorph names to be *Phomopsis phaseoli* and *D. phaseolorum*, respectively. However, Hobbs et al. (1985) considered *P. phaseoli* to be *nomen dubium* because the description of *P. phaseoli* by Petch (1922) might have been based on an immature specimen or could represent a separate entity as the alpha-conidia measurements by

Petch are small compared to measurements of other *Phomopsis* spp. on legumes. Additionally, *P. phaseoli* Petch is a later homonym of *P. phaseoli* (Desm.) Sacc; therefore, the former name is invalid according to Article 53.1 of the International Code of Nomenclature for algae, fungi, and plants (ICN). Kulik (1984) also provisionally named the anamorph and teleomorph of *D. phaseolorum* var. *caulivora Diaporthe phaseolorum* f. sp. *caulivora* and *P. phaseoli* f. sp. *caulivora*, respectively, because Koch's postulates were not yet satisfied for this variety by a non-wounding method of infection akin to how the fungus infects soybean naturally, viz., spraying the host with ascospores or conidia. However, stem canker symptoms have been obtained by spraying soybean with *D. phaseolorum* var. *caulivora* ascospores or conidia (Black et al. 1996; Ploetz and Shokes 1985). Santos et al. (2011) elevated *D. phaseolorum* var. *caulivora* to the species level, i.e., *Diaporthe caulivora*, because it was genetically distant to a *D. phaseolorum* isolate arbitrarily decided by van Rensburg et al. (2006) to be a reference isolate. Higley and Tachibana (1987) conducted pathogenicity tests and discovered that isolates of *D. phaseolorum* var. *caulivora* from Iowa and Mississippi caused different reactions on cultivars adapted to either state. They proposed that the Iowa and Mississippi isolates be designated race 1 and race 2, respectively (Higley and Tachibana 1987). Morgan-Jones (1989) proposed that the name *Diaporthe phaseolorum* f. sp. *meridionalis* be used for the causal organism of stem canker in the South, but this proposal was invalid because there was no Latin description of the fungus or a reference to a published Latin description, which was a requirement between 1935 and 2011 according to Article 39.1 of the ICN. Fernández and Hanlin (1996) validly named this organism *Diaporthe phaseolorum* var. *meridionalis*. van Rensburg et al. (2006) renamed *Diaporthe phaseolorum* var. *meridionalis Diaporthe aspalathi* because this fungus was genetically distant to a *D. phaseolorum* isolate arbitrarily decided by them to be a reference isolate. Kmetz et al.

(1974) isolated an unidentified species of *Phomopsis* from soybean cotyledons, stems, petiole and leaf debris, pods, and seeds that only produced pycnidia in culture. After doing an epidemiological study of seed infection in soybean, Kmetz et al. (1978) decided that because the undescribed *Phomopsis* sp. caused most of the soybean seed decay and *D. phaseolorum* var. *sojae* and *D. phaseolorum* var. *caulivora* have a *Phomopsis* anamorph, the disease should be called Phomopsis seed decay. Hobbs et al. (1985) named this undescribed species *Phomopsis longicolla*. Santos et al. (2011) gave *P. longicolla* the synonym *Diaporthe longicolla* but did not find or describe any sexual structures of the fungus. They also found a novel fungal species on soybean seed and named it *Diaporthe novem*. Finally, they gave *D. phaseolorum* the synonym *Phomopsis camptothecae* (Chang et al. 2005) because of 100% identity between the ITS sequences of two isolates of these two fungi. Gomes et al. (2013) made *D. sojae* the synonym of *P. longicolla* and *D. phaseolorum* var. *sojae* based on genetic similarity, disease etiology, and the sharing of a common host.

Distribution and host range of the *Diaporthe*/*Phomopsis* complex

The members of the DPC have a wide host range that includes economically important crops, including soybean, and various weeds and have been found all over the world. Other than the United States, members of the DPC have been found in Australia (Stovold and Francis 1987), Argentina (Pioli et al. 1997), Bolivia (Jaccoud-Filho et al. 1997), Brazil (Bolkan et al. 1976), Canada (Koch and Hildebrand 1943), China (Cui et al. 2009), Croatia (Vratarić et al. 1998), Ethiopia (Mengistu and Sinclair 1979), Ghana (Asante et al. 1998), Hungary (Szili 1975), Italy (Mannerucci and Gambogi 1978), Japan (Wolf and Lehman 1926), Kenya (Wanyera 2002); Malaysia (Raeisi et al. 2011), Romania (Hulea et al. 1973), Serbia (Jasnić and Vidić 1983), South Africa (Hamman et al. 1996), South Korea (Oh 1988), Taiwan (Wu and Lee 1985),

Tanzania (Jaccoud-Filho et al. 1997), and Uruguay (Sato et al. 1993). Additionally, soybean seed shipped to India from Poland, North Korea, Nigeria, South Korea, the former Soviet Union, Taiwan, Thailand, the United States, and Zimbabwe for use as germplasm were found to be infected with *D. phaseolorum* var. *sojae* (Agarwal et al. 1990; Agarwal et al. 2006). Likewise, soybean seed shipped from the United States to Greece was found to be infected with members of the DPC (Holevas et al. 2000).

The host range of *D. phaseolorum* var. *sojae* includes snap bean (*Phaseolus vulgaris* L.), cowpea (*Vigna unguiculata* (L.) Walp.), lima bean (*Phaseolus lunatus* L.), peanut (*Arachis hypogaea* L.), *Lespedeza* spp. Michx., Korean lespedeza (*Lespedeza stipulacea* Maxim.), wild bean (*Strophostyles helvola* (L.) Elliott), blue lupine (*Lupinus angustifolius* L.), pepper (*Piper* spp. L.), tomato (*Solanum lycopersicum* L.), okra (*Abelmoschus esculentus* (L.) Moench), onion (*Allium cepa* L.), garlic (*Allium sativum* L.) (Luttrell 1947), jack bean (*Canavalia ensiformis* (L.) DC.), chickpea (Cicer arietinum L.), sweet potato (*Ipomoea batatas* (L.) Lam.), perennial pea (*Lathyrus latifolius* L.), flat pea (*Lathyrus sylvestris* L.), lentil (*Lens culinaris* Medik.), birdsfoot trefoil (*Lotus corniculatus* L.), runner bean (*Phaseolus coccineus* L.), garden pea (*Pisum sativum* L.), fenugreek (*Trigonella foenum-graecum* L.), faba bean (*Vicia faba* L.), azuki bean (*Vigna angularis* (Willd.) Ohwi & H. Ohashi), mung bean (*Vigna radiata* (L.) R. Wilczek var. *radiata*), black-eyed pea (*Vigna unguiculata* (L.) Walp. subsp. *unguiculata*), white moneywort (*Alysicarpus vaginalis* (L.) DC.), common lespedeza (*Lespedeza striata* (Thunb. ex Murr.) Hook. & Arn.), tepary bean (*Phaseolus acutifolius* A. Gray), hemp sesbania (*Sesbania exaltata* (Raf.) Rydb. ex A.W. Hill), red clover (*Trifolium pratense* L.) (Kulik 1984), and a mangrove species (*Kandelia candel* (L.) Druce (Cheng et al. 2008). *P. longicolla* has been found on cowpea (*Vigna unguiculata* (L.) Walp.) (Roy and Ratnayake 1997), Valencia peanut (*Arachis hypogaea* L.)

(Sanogo and Etarock 2009), and eggplant (*Solanum melongena* L.) (Shu et al. 2014). *D. phaseolorum* var. *meridionalis* has been found on blue lupin (*Lupinus angustifolius* L.) (Yorinori 1990) and rooibos (*Aspalathus linearis* (Burm. f.) R. Dahlgren) (van Rensburg et al. 2006). P. *camptothecae*, a synonym of *D. phaseolorum*, was found on happy tree (*Camptotheca acuminata* Decne) (Chang et al. 2005; Santos et al. 2011). *Diaporthe* spp. and *Phomopsis* spp., including *P. longicolla*, *D. phaseolorum* var. *sojae*, and *D. phaseolorum* var. *caulivora* have also been found on a number of wild and purposely infected weeds, such as velvetleaf (*Abutilon theophrasti* Medik.) (Hepperly et al. 1980; Li et al. 2001; Vrandecic et al. 2005), spiny amaranth (*Amaranthus spinosus* L.), lion's tail (*Leonotis nepetaefolia* (L.) R.Br.), motherwort (*Leonurus sibiricus* L.) (Cerkauskas et al. 1983), hophornbeam copperleaf (*Acalypha ostryaefolia* Riddell), smooth pigweed (*Amaranthus hybridus* L.), redroot pigweed (*Amaranthus retroflexus* L.), common ragweed (*Ambrosia artemisiifolia* L.), peppervine (*Ampelopsis arborea* (L.) Koehne), sicklepod (*Cassia tora* L.), horseweed (*Conyza canadensis* (L.) Cronquist), woolly croton (*Croton capitatus* Michx.), crabgrass (*Digitaria* spp. Haller), eclipta (*Eclipta alba* (L.) Hassk.), annual fleabane (*Erigeron annuus* (L.) Pers.), spotted spurge (*Euphorbia maculata* L.), bitter sneezeweed (*Helenium amarum* (Raf.) H. Rock), camphorweed (*Heterotheca subaxillaris* (Lam.) Britton & Rusby), pitted morning glory (*Ipomoea lacunose* L.), tall morning glory (*Ipomoea purpure*a (L.) Roth), smallflower morning glory (*Jacquemontia tamnifolia* (L.) Griseb.), frogfruit (*Phyla nodiflora* (L.) Greene), Pennsylvania smartweed (*Polygonum pensylvanicum* L.), common purslane (*Portulaca oleracea* L.), kudzu (*Pueraria lobata* (Willd.) Ohwi), wild blackberry (*Rubus* sp. L.), curly dock (*Rumex crispus* L.), common groundsel (*Senecio vulgaris* L.), prickly sida (*Sida spinosa* L.), johnsongrass (*Sorghum halepense* (L.) Pers.), blue verbena (*Verbena hastata* L.), narrowleaf vetch (*Vicia angustifolia* L.), common cocklebur (*Xanthium*

strumarium L.) (Roy et al. 1994b), black nightshade (*Solanum nigrum* L.), northern jointvetch (*Aeschynomene virginica* (L.) Britton, Sterns & Poggenb.), entireleaf morning glory (*Ipomoea hederacea* Jacq. var. *integriuscula* A. Gray), redweed (*Melochia corchorifolia* L.), hairy indigo (*Indigofera hirsuta* L.), wild poinsettia (*Euphorbia heterophylla* L.) (Black et al. 1996), giant ragweed (*Ambrosia trifida* L.) (Roy et al. 1997), large crabgrass (*Digitaria sanguinalis* (L.) Scop.) (Jackson et al. 2002), nodding spurge (*Chamaesyce nutans* Lag.), Illinois bundle-flower (*Desmanthus illinoensis* (Michx.) MacMill. ex B.L. Rob. & Fernald), Texasweed (*Caperonia palustris* (L.) A. St.-Hil.), slender aster (*Aster exilis* Elliott), and prostrate knotweed (*Polygonum aviculare* L.) (Mengistu et al. 2007).

Cultural morphology of the members of the *Diaporthe*/*Phomopsis* complex

Potato dextrose agar (PDA) is the most common substrate on which members of the DPC are characterized and the morphological descriptions stated in this section are for fungi grown on PDA unless otherwise stated. *P. longicolla* can be differentiated from other members of the DPC by its lack of a known teleomorph, or sexual stage of the fungus (Hobbs et al. 1985), although Fernández and Hanlin (1996) recorded an instance where a *P. longicolla* isolate formed fertile perithecia on autoclaved elm bark, and Vidić et al. (2013) observed perithecia on soybean stems inoculated with a *P. longicolla* isolate, but single-ascospore isolates could not be obtained from them. *D. phaseolorum* var. *sojae* can form perithecia, although some isolates only produce pycnidia, but this might be substrate dependent (Athow and Caldwell 1954; Fernández and Hanlin 1996; Gerdemann 1954; Hildebrand 1954; Hildebrand 1956; Jaccoud-Filho et al. 1997; Kmetz 1975; Kurata 1960; Luttrell 1947; Pioli et al. 2003). Likewise, *D. phaseolorum* var. *caulivora* has been found at times to not produce perithecia (Fernández and Hanlin 1996; Kmetz 1975). *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var.

meridionalis have been found to produce perithecia both singly and in caespitose groups on various substrates (Athow and Caldwell 1954; Fernández and Hanlin 1996; Lehman 1923; Luttrell 1947). The most defining characteristic of *P. longicolla* is that it forms long pycnidial beaks ranging from 200 to 500 µm in length, hence the epithet longicolla (longus [long] + collum [neck]). If *D. phaseolorum* var. *sojae* has pycnidial beaks at all, they are shorter than 200 µm. Likewise, *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* have been reported to have short or mutic, or unbeaked, pycnidia (Hobbs et al. 1985; Jaccoud-Filho et al. 1997; Jasnić and Vidić 1983; Pioli et al. 2003). However, Luttrell (1947) found long pycnidial beaks produced only on *D. phaseolorum* var. *sojae* isolates that also formed perithecia. The conidiophores of *P. longicolla* are simple or frequently branched while the conidiophores of *D. phaseolorum* var. *sojae* are simple or rarely branched (Athow and Caldwell 1954; Hobbs et al. 1985; Lehman 1923). It has been reported that some *Diaporthe* spp. form conidia on conidiophores outside pycnidia, i.e., on exposed mycelia (Hildebrand 1954). The two most common types of conidia formed by species of the genus *Phomopsis* are alpha and beta-conidia. Alpha-conidia are hyaline, aseptate, fusiform, straight, and usually biguttulate. Beta-conidia are hyaline, aseptate, filiform, straight or more often hamate, and eguttulate (Sutton 1980). However, asexual spores intermediate between alpha and beta spores, also called gamma conidia, have been found among the DPC by some researchers (Hildebrand 1954; Luttrell 1947; Wehmeyer 1933; Udayanga et al. 2011). Beta-conidia are thought to possibly be either functional or relictual spermatia, and attempts to germinate beta-conidia in the DPC members have yet to be fruitful (Hildebrand 1954; Jensen 1983; Lehman 1923; Morgan-Jones 1985; Wehmeyer 1933). The frequency of beta-conidia differentiates *P. longicolla* from *D. phaseolorum* var. *sojae* in that the former rarely produces them while in the latter they are common (Hobbs et al. 1985).

Furthermore, while subculturing *P. longicolla* did not change the frequency of beta-conidia, subculturing *D. phaseolorum* var. *sojae* increased the frequency of beta-conidia (Kmetz 1975). However, *P. longicolla* isolates exposed to low temperatures for a week started to produce beta-conidia, some even exclusively, but this was not a characteristic shared by all isolates, with one *P. longicolla* isolate only forming alpha-conidia whether exposed to low temperatures or not (Vidić et al. 2013). Additionally, *D. phaseolorum* var. *sojae* isolates that do not develop perithecia have been found to produce only alpha-conidia (Luttrell 1947). *D. phaseolorum* var. *meridionalis* has been found to only form alpha-conidia (Fernández and Hanlin 1996). Alpha-conidium size cannot be used to differentiate *P. longicolla*, *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* as the measurements overlap (Chao and Glawe 1985; Frosheiser 1957; Hobbs et al. 1985; Sato et al. 1993). However, alpha-conidia of *P. longicolla* and *D. phaseolorum* var. *meridionalis* have been reported by some to be irregular or variable in shape (Jaccoud-Filho et al. 1997; Morgan-Jones 1985). Conidial exudation from pycnidia in a globular gelatinous matrix has been found to be common among *P. longicolla* but rare among *D. phaseolorum* var. *sojae* (Kmetz et al. 1975). *Phomopsis* spp. have also been found to exude conidia in a tendril-like manner (Hildebrand 1954; Sun et al. 2012a). Chlamydospores, or hyphae-derived resting spores, have been found to be produced by *D. phaseolorum var. meridionalis* and chlamydospore-like cells have been found to be produced by *P. longicolla* (Morgan-Jones 1985; Sato et al. 1993). *P. longicolla* can be differentiated from *D. phaseolorum* var. *sojae* by stroma morphology with the former forming large, effuse stromata and the latter forming scattered, pulvinate, or small cushion-shaped, stromata, although Chao and Glawe (1984) found *P. longicolla* to form pulvinate stromata. (Athow and Caldwell 1954; Hobbs et al. 1985; Pioli et al. 2003). *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var.

meridionalis have been shown to form both small and large stromata depending on the isolates inspected (Chao and Glawe 1984; Fernández and Hanlin 1996; Pioli et al. 2003). The mycelia, color of the mycelia, and color of the reverse, or underside, of the PDA plates on which the fungi are growing on are very diverse within the DPC (Athow and Caldwell 1954; Chao and Glawe 1984; Chao and Glawe 1985; Fernández and Hanlin 1996; Hobbs et al. 1985; Jaccoud-Filho et al. 1997; Kmetz 1975; Lehman 1923; Pioli et al. 2003; Sato et al. 1993). The mycelia of *P. longicolla* have been described as floccose and dense. The mycelia of *D. phaseolorum* var. *sojae* have been described as floccose, ropy, cottony, tufted, or felty. The mycelia of *D. phaseolorum* var. *caulivora* have been described as compact, shrunken, fluffy with age, floccose with knots, cottony, tufted, or felty. The mycelia of *D. phaseolorum* var. *meridionalis* have been described as lanose, cottony, or tufted. In terms of the color of the mycelia, *P. longicolla* is white with occasional green and/or yellow areas, *D. phaseolorum* var. *sojae* is white to gray with occasional yellow and/or green areas, *D. phaseolorum* var. *caulivora* is white or white-yellow, and *D. phaseolorum* var. *meridionalis* is white or white-buff or tan. With age, the mycelia color of *P. longicolla* can have greenish-yellow or a grayish shade, the mycelial color of *D. phaseolorum* var. *sojae* can turn tan to brown, the mycelial color of *D. phaseolorum* var. *caulivora* can turn ochre, and the mycelial color of *D. phaseolorum* var. *meridionalis* can turn tan or light to dark brown. In terms of reverse color, *P. longicolla* is white, *D. phaseolorum* var. *sojae* is yellow, grayish, tan to dark brown, or black, *D. phaseolorum* var. *caulivora* is light ochre to tan, light yellow, or yellow, and *D. phaseolorum* var. *meridionalis* is light brown to tan. With age, the reverse color of *D. phaseolorum* var. *caulivora* can turn yellow. Additionally, the agar medium on which *D. phaseolorum* var. *sojae* is growing on can darken with or without age.

Identification of the *Diaporthe*/*Phomopsis* complex

There are a variety of ways that members of the DPC can be identified. The most common method is an agar plate bioassay in which seed are plated on an agar medium (e.g., potato dextrose agar), and the fungus is identified by mycelial morphology, spore type, and other morphological traits after a certain incubation time (Gleason et al. 1987). A modification of this method uses a medium containing four fungicides that allows *D. phaseolorum* var. *caulivora* to grow while completely inhibiting growth by *D. phaseolorum* var. *sojae* (Phillips 1984).

Non-specific detection of any member of the DPC is possible using polymerase chain reaction (PCR) with the primers Phom I and Phom II, which amplify a region within two internal transcribed spacers (ITS) (Zhang et al. 1997). Additionally, primers DphLe and DphRi have been developed to differentiate *D. phaseolorum* var. *meridionalis* from *D. phaseolorum* var. *sojae* and *Diaporthe* spp.; however, *D. phaseolorum* var. *caulivora* was not tested using these primers (Vechiato et al. 2006). Three TaqMan® primer and probe sets have been developed for identification of (i) only *P. longicolla*, (ii) only *D. phaseolorum* var. *caulivora*, and (iii) *P. longicolla*, *D. phaseolorum* var. *sojae*, and *D. phaseolorum* var. *meridionalis* (Zhang et al. 1999). Therefore, the TaqMan primer and probe sets cannot distinguish *D. phaseolorum* var. *sojae* and *D. phaseolorum* var. *meridionalis* from the other DPC members. A PCR-restriction fragment length polymorphism (PCR-RFLP) assay with ITS4 and ITS5 primers and *Alu*I, *Rsa*I, *Hha*I, *Mse*I, and *Scr*FI restriction enzymes can be used to distinguish members of the DPC from each other (Zhang et al. 1998). Additionally, random amplified polymorphic DNA (RAPD) markers have been shown to be effective in differentiating between the four DPC members and *D. phaseolorum* var. *meridionalis* in particular (Jaccoud-Filho et al. 1997; Mengistu and Ray 2004).

An indirect enzyme-linked immunosorbent assay (ELISA) of soybean seed coats using antibodies to *P. longicolla* has been shown to be useful in identifying the severity of infection of DPC members while a seed immunoblot assay (SIBA) of soybean seed has been shown to be a reliable alternative to the agar plate bioassay, with the incidence of appearance of blue color on nitrocellulose paper after seed incubation correlating to incidence of infection measured by the agar plate bioassay (Gleason et al. 1987). Strong cross-reactivity to *D. phaseolorum* var. *sojae* and *D. phaseolorum* var. *caulivora* was detected in indirect ELISA, while cross-reactivity to *D. phaseolorum* var. *sojae* and *Chaetomium* sp. was detected with SIBA. Double-antibody sandwich ELISA (DAS-ELISA) using antibodies against *P. longicolla* has been used to quantify the relative amount of *P. phaseoli* on soybean stems between nodes one and two in growth stages V2, V3, and V4 (Velicheti et al. 1993). Strong cross-reactivity to *P. phaseoli*, *D. phaseolorum* var. *caulivora, D. phaseolorum* var. *meridionalis,* and *Colletotrichum truncatum* was detected by immunoblot analysis (Velicheti et al. 1993). DAS-ELISA was found to be more specific, and 100 times more sensitive than indirect ELISA (Brill et al. 1994). Additionally, polyclonal antibodies against a culture filtrate of *P. longicolla* were more specific but less reactive than polyclonal antibodies against a mycelial extract of *P. longicolla* (Brill et al. 1994). Indirect ELISA has also been used for *D. phaseolorum* var. *caulivora* isolates but *Phomopsis* spp. cross-reactivity exists, and using this method to test seed for *D. phaseolorum* var. *caulivora* has been unsuccessful (Shelby 1985).

In addition to qualitative methods to detect the members of the DPC on soybean seed, quantitative methods also exist. Ultrasound analysis can be used to determine *P. longicolla* infection of asymptomatic soybean seeds (Walcott et al. 1998). The process involves dropping a seed on a piezoelectric transducer that generates a sound wave that is converted into an electrical

impulse signal that is sent to a voltage digitizer that converts the analog signal to a digital signal. The digital signal is then analyzed by a computer program and the magnitude of the peak, slope, and width of the electrical impulse signal is quantified. Asymptomatic *P. longicolla* infection is negatively correlated with the peak values, which are representative of seed weight, but poorly positively correlated to slope and width values, which are representative of seed softness. Red, green, and blue color analysis of soybean seeds can also be used to identify symptomatic soybean seeds infected with *Phomopsis* spp., although the accuracy is only 40.9% when asymptomatic seeds, materially damaged seeds, or seeds with colors other than beige were tested (Ahmad et al. 1999) or 66% when symptomatic seeds were tested (Paulsen et al. 1989). Further, accuracy of symptomatic *Phomopsis* spp. infection was 74% and 77% when the soybean seed for a particular year were tested against a training set developed from exemplar seed harvested from the same year with color and sphericity being the metrics for identification (Casady et al. 1992). Near-infrared spectroscopy has been used to develop five-class neural network models consisting of healthy seed and fungal-damaged seed that can be used to classify *Phomopsis*-damaged seed with 99% accuracy (Wang et al. 2004). Neural network models consist of a wide array of models that work like the human brain in that they need to be trained beforehand with supervision to give an output for a given input (Wang et al. 2002). Ergosterol, a membrane sterol specific to fungi and absent from higher plants, content has been found to be positively correlated to the fungal dry mass of *D. phaseolorum*, allowing for the measurement of the intensity of colonization in a particular seed (Xue et al. 2006). Electrical conductivity of seed leachate has not been found to be associated with PSD (Loeffler et al. 1988; Thomison et al. 1989).

Disease cycle of Phomopsis seed decay

P. longicolla and *D. phaseolorum* var. *sojae* overwinter on infected seed and as pycnidia on soybean crop residue in the field (Garzonio and McGee 1983; Kmetz et al. 1979; Lehman 1923). Infected seed, however, are not a major source of stem, pod, or seed infection, even though infected seed can result in infected seedlings (McGee et al. 1980; Garzonio and McGee 1983). Incidence of pod and stem blight symptoms is only an indicator of PSD in certain cultivars, which makes it an unreliable method to estimate PSD incidence (Hepperly and Sinclair 1980a; Kmetz et al. 1978; Prasartsee et al. 1975). *P. longicolla*, *D. phaseolorum* var. *sojae*, or *D. phaseolorum* var. *caulivora* stem and pod infection and *P. longicolla* leaf infection are not correlated with PSD, although Mengistu et al. (2009) did find *P. longicolla* pod infection to have a low but significant positive correlation with PSD while Bisht and Sinclair (1985) found *D. phaseolorum* var. *sojae* pod and stem infection to be significantly negatively correlated with PSD (Mengistu et al. 2009; Xue et al. 2007). Prediction of PSD incidence at harvest maturity (R8) using pod infection at R6 seems to be possible but only when wet, warm weather occurs after pod set (Jardine 1991; McGee 1986). Seed are thought to function as vectors through which *P. longicolla* and *D. phaseolorum* var. *sojae* are spread to new places (Garzonio and McGee 1983; Lehman 1923). Conidia from pycnidia on overwintered soybean straw act as the primary inoculum while dead non-overwintered soybean stems do not form mature pycnidia that exude any conidia (Kmetz et al. 1979; Kulik 1984; Lehman 1923). In Maryland, based on incubated dead soybean stems, the time of conidial exudation from pycnidia starts around the beginning of May and ends around the end of July (Kulik 1984); however, exuding pycnidia were found as late as mid-November on incubated mature soybean stems in Ontario (Hildebrand 1954). This is thought to be the reason why mature plants from infected and uninfected seed grown on

continuous soybean fields tend to be more severely infected compared to plants in fields with corn-soybean rotations or continuous corn regimens (Garzonio and McGee 1983). Fallen cotyledons and petioles and dead soybean stem pieces from the current season, but not fallen soybean leaves, form pycnidia that later form conidia that act as secondary inoculum (Crittenden et al. 1967; Kmetz et al. 1979). Conidia of *D. phaseolorum* var. *sojae* attach themselves on soybean unifoliate leaves and cotyledons by reticula and penetrate via stomata with or without forming appressoria and not via the cuticle (Kulik 1988). Mycelia of *D. phaseolorum* var. *sojae*-infected soybean stems, which colonize the pith and epidermis but hardly colonize the sieve elements and do not colonize the vascular tissue, have likewise been shown to produce appressoria on the epidermis (Hill et al. 1981). Furthermore, penetration of *P. longicolla*-infected pods is also preceded by formation of appressoria (Baker et al. 1987). Splashing water is the main mode by which conidia are transferred from pycnidia on soybean straw to soybean plants, although conidia exist in the air as well (Kmetz et al. 1979; Wu and Lee 1985).

Inclement weather and insects, both of which can wound pods, can provide a way for the conidia to infect a plant, even if it is resistant to PSD. Artificial pod wounding has been shown to significantly increase PSD incidence even in a PSD-resistant plant introduction (Hepperly and Sinclair 1980a; Roy and Abney 1988). Stink bug-damaged seed and corn earworm-damaged seed have been shown to have significantly greater seed with PSD (Crittenden 1968; França Neto et al. 1985). However, Russin et al. (1988) found no significant difference between stink bug population level (a higher level is associated with a higher percentage of stink bug-damaged seed) and PSD, although a significant increase in PSD was found in seed harvested three weeks after harvest maturity from the upper halves of plants with a moderate stink bug population level. Additionally, although Jones et al. (2011) found a significantly higher occurrence of fungi in

stink bug-damaged seed, incidence of PSD was not significantly different in stink bug-damaged seed (J. Gore, personal communication). Moreover, Lima bean pod borer-damaged pods have been shown to contain seed with significantly decreased levels of PSD than non-damaged seed (Hepperly 1985).

PSD can begin as early as R3 (i.e., the beginning pod stage) in the seed from pods in the lower half of the plant but does not increase dramatically until R7 (i.e., physiological maturity) (Kmetz et al. 1978; Ploper et al. 1992; Tomes et al. 1985). Seed infection by *P. longicolla* and *D. phaseolorum* var. *sojae* was first thought to be the result of systemic infection, but later, evidence surfaced that showed that it was the result of local infection. *D. phaseolorum* var. *sojae* was initially thought to infect seed via systemic infection because seed in the distal pod locules were infected less often than those in the proximal pod locules (Kilpatrick 1957). It was later reported that seed from the middle and distal pod locules were infected with *P. longicolla* as often as seed from the proximal pod locules, supporting the idea of a local infection (Baird et al. 2001; Kmetz et al. 1978; Roy and Abney 1988; Tomes 1985). An association between pod locule infection with *P. longicolla* and seed infection in the same locule, as well as the absence of *P. longicolla* infection in pods and petioles near stem points inoculated with *P. longicolla*, further supports the idea of local infection (Kmetz et al. 1979; Tomes et al. 1985). Additionally, the absence of *P. longicolla* infection on the main stem axis where attached cotyledons were infected with *P. longicolla* corroborates this idea (Kmetz et al. 1978; Kmetz et al. 1979). It should be noted, however, that with enough relative humidity and time, *P. longicolla* was recovered from the cotyledonary node in plants with a hypocotyl infection (Kmetz et al. 1979). Inoculation of soybean at the vegetative stages produced little to no pod infection at R6 (i.e., fully developed seed) compared to inoculation at the reproductive stages (Lamka and McGee 1986). The

protection of pods with bags with or without additional spraying of Dithane M-45 (mancozeb) fungicide significantly reduced *D. phaseolorum* var. *sojae* infection on seed, although seed infection was still existent (Athow and Laviolette 1973; Wu and Lee 1985). Therefore, it seems as though seed infection can occur locally or systemically with local infection being the predominant mode of infection.

P. longicolla and *D. phaseolorum* var. *sojae* can colonize all parts of the seed. The seed coats of mature *D. phaseolorum* var. *sojae*-infected seed have mycelia colonizing the osteosclereid, or hourglass, cell layer most abundantly with less colonization in the parenchyma cell layer and even less in the macrosclereid, or palisade, cell layer (Begum et al. 2008; Ilyas et al. 1975; Wu and Lee 1985). In immature *P. longicolla*-infected seed, the osteosclereids, parenchyma, endothelium, and cotyledons contained abundant mycelia (Baker et al. 1987). *P. longicolla* and *D. phaseolorum* var. *sojae* mycelia seem to disintegrate osteosclereids, possibly because of their high starch content, and the aleurone and parenchymatous cells of the endosperm (Baker et al. 1987; Ilyas et al. 1975; Schneider et al. 1974; Singh and Sinclair 1986). In addition to seed coat colonization, *P. longicolla* and *D. phaseolorum* var. *sojae* colonize seed embryos at a rate similar to their colonization of seed coats in mature seed (Bolkan 1976; Ilyas et al. 1975). Furthermore, Wu and Lee (1985) found *D. phaseolorum* var. *sojae* to colonize the seed coat, cotyledons, and embryonal axis at a ratio of 2.68:2.06:1 in mature seed. However, Franca Neto and West (1989) found embryo colonization by *P. longicolla* and *D. phaseolorum* var. *sojae* to be rare compared to seed coat colonization by these fungi. Apart from the seed coat, *P. longicolla* mycelia have been found between the seed coat and plumule, hypocotyl-radicle axis, and cotyledon, between the two cotyledons, in the cotyledon cell walls, and in a few upper cell layers of the hypocotyl-radicle axis, but no hyphae were found in the vascular elements of the cotyledons (Singh and

Sinclair 1986). Moreover, those cotyledonary cells colonized by *P. longicolla* were hypertrophied with decreased cytoplasm and increased vacuole sizes. Additionally, pycnidial initials have been found to form in the parenchyma and endothelium tissues of immature seed (Baker et al. 1987). In mature *D. phaseolorum* var. *sojae*-infected seed incubated under high relative humidity and temperature, pycnidia were found on outer or inner surfaces of the epidermis with the ostioles facing inward toward the cotyledons, on the palisade layers of the hilum, on the root cap, and on the cotyledon-embryo axis, but no pycnidia formed between the seed coat and the cotyledons or between the cotyledons at the suture even though mycelial mats developed in these areas (Rodriguez-Marcano and Sinclair 1978). The palisade layers of the hilum were covered with pycnidia, but abundant mycelial growth was not observed. Furthermore, Begum et al. (2008) found pycnidia in the palisade, hourglass, and parenchyma cell layers of mature *D. phaseolorum* var. *sojae*-infected seed incubated on moist blotter paper. However, they did not find any hyphae or pycnidia on the cotyledonary or embryonic tissue. A high concentration of *P. longicolla* hyphae in the hilar region of the seed coat and in the upper palisade cell layer beneath the hilar region as well as hyphal aggregation in the stellate parenchyma of the hilar region and the hilar tracheids (Roy and Abney 1988; Singh and Sinclair 1986) suggest that seed infection occurs through the funiculus, although Rodriguez-Marcano and Sinclair (1978) found no abundant mycelial growth in the palisade layers of the hilum and no mycelia at all in the hilar tracheids and Kunwar et al. (1985) similarly found no mycelia in the hilar region and stellate parenchyma. Furthermore, hyphae have been found in other regions of the seed coat, and penetration through the pores on the seed coat of mature and immature seed and through the micropyle of mature seed has been observed by *P. longicolla* and *D.*

phaseolorum var. *sojae*, showing that direct seed infection can also occur (Baker et al. 1987; Roy and Abney 1988; Yaklich and Kulik 1987; Yelen and Crittenden 1967).

Localized pod infections by *D. phaseolorum* var. *sojae* seem to occur either between trichomes and their surrounding epidermal cells or through pod stomata (Hepperly and Sinclair 1980a; Yaklich and Kulik 1987); however, Baker et al. (1987) did not observed *P. longicolla* penetration through pod stomata. Cultivars and lines with greater pod pubescence or less stomata were found to be more resistant to PSD than those with less pod pubescence or more stomata (Anderson et. al 1995; Hepperly and Sinclair 1980a). It has been shown that pod pubescence has the ability to trap a great number of *P. longicolla* conidia, reducing the total amount of *P. longicolla* conidia that end up on the pod surface where pod penetration and subsequent seed colonization would be most parsimonious (Baker et al. 1987). However, nearly-isogenic lines of Clark and Harosoy differing in pubescence density did not show any difference in PSD incidence in the majority of environments in which they were tested (Thomison et al. 1993).

Epidemiology of Phomopsis seed decay

Relative humidity, temperature, and rainfall seem to be the three most important environmental factors responsible for PSD development. Spilker et al. (1981) found that PSD incidence was greatest when high relative humidity and high temperature conditions were imposed at R6 on soybeans inoculated at R4. Plants under high relative humidity and low temperature had moderate but reduced PSD, and plants under low relative humidity and high or low temperature had almost no PSD. Shortt et al. (1981) found that PSD was positively correlated with total rainfall during August, September, and October in Illinois but weakly positively correlated with temperature, and TeKrony et al. (1996) similarly found a significant positive correlation between

rainfall from R5 to R8 and PSD. On the other hand, TeKrony et al. (1983) found that total and daily rainfall between either R5 through R7 or R7 through R8 were not correlated with PSD. They also found that minimum relative humidity and temperature under the two developmental periods were significantly but weakly correlated with PSD. Balducchi and McGee (1987) found a strong positive correlation between PSD and temperature under field conditions where plants were inoculated at R5 and in the laboratory where detached pods naturally infected with *P. longicolla*, *D. phaseolorum* var. *sojae*, and *D. phaseolorum* var. *caulivora* and detached pods inoculated with *P. longicolla* were kept at 100% relative humidity for one to seven days. They also found that seed from pods inoculated at R6 with *P. longicolla* had more PSD when they were detached and experienced high relative humidity at R7 than if the pods were detached and experienced high relative humidity at R8. Moreover, they found that a minimum of three continuous days of 100% relative humidity was necessary for seed in detached pods that were inoculated by immersion in a suspension of *P. longicolla* to have a seed infection rate of more than 90% and that any interruption in this period would result in substantially less PSD.

The reason high relative humidity and rainfall play an important role in PSD is because those conditions keep the pods at a high water content level and pod water content level is proportional to seed infection rate (Rupe and Ferriss 1986). In agreement with this theory, the rate of moisture loss from pods and seed at R7 to R8 was shown to be negatively correlated PSD while the R7 to R8 growth stage interval was shown to be positively correlated with PSD (Ploper et al. 1992). Meanwhile, Vaughan et al. (1989) found duration of R6 to R7 to be correlated to PSD. Similarly, the use of growth regulators to either decrease the duration of R6 to R7 or increase the duration of R5 to R7 led to a respective decrease and increase of PSD (Abney et al. 1984). When a hybrid soybean population segregating for male sterility had its male-fertile plants depodded, the male-

sterile and depodded male-fertile plants had greater PSD compared to untreated male-fertile plants (Vaughan et al. 1989). Thomison et al. (1988) found depodded cultivars with one pod per node to have increased PSD compared to non-depodded cultivars. Depodding has been shown to delay maturity, and male-sterile plants have low pod set and delayed maturity (Brim and Young 1971; Leopold et al. 1956; Thomison et al. 1988). Delaying maturity would increase the R6 to R7 growth stage, the R7 to R8 growth stage, or both, explaining the greater seed infection levels found.

Other environmental factors also affect PSD. Irrigation has been shown to increase PSD (Anderson and Buzzell 1985; Balducchi and McGee 1987; Kulik and Yaklich 1991; Mengistu et al. 2009; Mengistu and Heatherly 2006; Ross 1975). Additionally, areas near rivers and coasts experience higher humidity levels, and soybeans grown in those areas have more PSD (Shortt et al. 1981; Stovold and Francis 1987). Altitude might also play a role in PSD with lower altitudes having greater PSD (Kim et al. 1996). Increased ultraviolet-B radiation has also been shown to increase PSD (Webb and Biggs 1984). Additionally, delay in harvest increases PSD (Athow and Laviolette 1973; Ellis and Sinclair 1976; Kim et al. 1996; Kmetz et al. 1978; Mengistu et al. 2010; Ross 1975; Sij et al. 1985; TeKrony et al. 1996; Thomison et al. 1990; Thomison et al. 1993; Wilcox et al. 1974).

Early maturing soybeans are more vulnerable to PSD, not because they are genetically susceptible, but because they mature during periods when high relative humidity, rain, and warm weather are more likely (Anderson and Buzzell 1985; Gillen et al. 2012; Kilpatrick 1957; Shortt et al. 1981; TeKrony et al. 1984). An early maturity group is relative to the latitude of a location; for example, in the Mid-South, where the Early Soybean Production System (ESPS) is used, a maturity group of III or IV would be considered early while in Illinois, a maturity group of I or II

would be considered early (Mayhew and Caviness 1994; Shortt et al. 1981). The ESPS makes use of early planting of earlier-than-normal maturity group cultivars to escape drought during the water-intensive reproductive stages of soybean development, but this also means that the soybeans will mature during high temperature and humidity, conditions ideal for PSD (Heatherly and Bowers 1998). Early-maturing, determinate and semi-determinate, and late-flowering isolines of the cultivars Clark and Harosoy had more PSD than late-maturing, indeterminate, normal-flowering and delayed flowering isolines (Thomison et al. 1990; Vaughan et al. 1989). Other soybean near-isogenic lines derived from a variety of cultivars that segregate for maturity loci alleles show the same relationship, i.e., later-maturing isolines have decreased PSD (Balles and Abney 1983). Likewise, a significant negative correlation has also been found between days to maturity and PSD in recombinant inbred lines (RILs) segregating for PSD resistance in the greenhouse (Sun et al. 2013). However, when 'Clark' and 'Harosoy' isolines were planted using an ESPS in mid-April in Mississippi and matured during hot and humid conditions, there were no significant differences among the isolines with differing maturities for PSD in each cultivar set in the two years tested, except for one isoline in one year that experienced a significant rain event prior to maturity (Gillen et al. 2012). Likewise, when favorable environmental conditions occurred during maturation, the effect of stem determination on PSD disappeared or was reduced (Thomison et al. 1990). Similarly, no correlation was found between maturity and PSD in soybean genotypes grown in the tropics due to constant climatic conditions (Paschal and Ellis 1978). Flowering time was confounded with maturity; therefore, it was not possible to contrast normal- and delayed-flowering isolines with late-flowering isolines (Thomison et al. 1990). Additionally, when the isolines tested by Vaughan et al. (1989) and other cultivars and lines were stagger planted, the early-planted soybeans had more PSD than the middle- and/or late-

planted soybeans (Grau and Oplinger 1981; Kim et al. 1996; TeKrony et al. 1984; TeKrony et al. 1996; Vaughan et al. 1989; Wrather et al. 1996; Wrather et al. 2003). Moreover, soybeans of early and late maturity groups subjected to an artificial photoperiod of 10 hours before flowering, but returned to a normal photoperiod after flowering, matured earlier and had more PSD than their counterparts that had not been subjected to an artificial photoperiod before flowering (Wilcox et al. 1985).

Seed from pods in the lower portions of the plant experience greater *P. longicolla*, *D. phaseolorum* var. *sojae*, and *D. phaseolorum* var. *caulivora* seed infection than seed from pods in the upper portions of the plant (Hepperly and Sinclair 1980a; Kmetz et al. 1978; Thomison et al. 1987; Thomison et al. 1988). However, Kmetz et al. (1978) found the opposite to be true for *D. phaseolorum* var. *sojae* seed infection. Similarly, Jeffers et al. (1982) found *Diaporthe* spp. to more likely infect the seed from pods in the lower portions of the plant, although the total *Diaporthe* spp. seed infection level was very low (≤6%). The likelihood of PSD increases when plants are grown on high-moisture soil compared to low-moisture soil (Thomison et al. 1987). Likewise, seed from pods of lodged branches in contact with the soil have significantly more PSD than seed from pods of erect stems (Wilcox and Abney 1971). Lodging by itself, however, is not associated with increased PSD (Garzonio and McGee 1983; Wilcox and Abney 1971). It would seem as though increasing plant height would alleviate PSD due to less pods being closer to the soil, although this has yet to be tested without confounding factors (Thomison et al. 1990).

Hard-seeded cultivars and lines with impermeable seeds have been shown to be less susceptible to PSD (Kulik and Yaklich 1991; Roy et al. 1994a; Yaklich and Kulik 1987). Additionally, incidence of hard seed has been shown to be negatively correlated to PSD (Mengistu et al. 2009). About 60% of the variation in seed infection can be explained by impermeability; therefore,

some of the resistance to PSD is contributed by permeable seed of hard-seeded lines (Roy et al. 1994a). Within hard-seeded lines, impermeable seed show greater resistance to PSD than permeable seed (Hill et al. 1985). Contradicting these studies, Smith et al. (2008a) found a significant but very low positive correlation between incidence of hard seed and PSD.

Interaction between members of the *Diaporthe*/*Phomopsis* complex and other fungi and bacteria

Members of the DPC have been found to interact with a number of fungi and bacteria. Antagonism has been found between *Phomopsis* spp. and *Cercospora kikuchii* (Hepperly and Sinclair 1981; McGee et al. 1980; Pathan et al. 1989; Roy and Abney 1977). Yeh and Sinclair (1982) found cercosporin, the photosensitive phytotoxin produced by *C. kikuchii*, and culture filtrates of *C. kikuchii* to not be antagonistic with *P. longicolla*, but Velicheti and Sinclair (1992) did find an antagonistic relationship between cercosporin and *P. longicolla* and *D. phaseolorum* var. *sojae* when the test plates were exposed to light but not when they were incubated in the dark. Antagonism has also been found between *Phomopsis* spp. and *Fusarium* spp., but this interaction might be host dependent (McGee et al. 1980). *Colletotrichum truncatum* has been found to be antagonistic to *P. longicolla* (Hepperly et al. 1983). Furthermore, *Colletotrichum truncatum* and *Colletotrichum destructivum* have been found to suppress pycnidial development on incubated petioles from plants inoculated with either *Colletotrichum* sp. or with a combination of one of the *Colletotrichum* sp. and *D. phaseolorum* var. *sojae* compared with plants inoculated with only *D. phaseolorum* var. *sojae* (Manandhar et al. 1987a). Interestingly, while plants inoculated with either *Colletotrichum* sp. or with a combination of one of the *Colletotrichum* sp. and *D. phaseolorum* var. *sojae* did not have significantly greater seed infection by *D. phaseolorum* var. *sojae*, plants inoculated with *D. phaseolorum* var. *sojae* in combination with *C. truncatum* had significantly greater *C. truncatum* seed infection.

Chaetomium globosum has been found to suppress pycnidial development and extent of colonization by *D. phaseolorum* var. *meridionalis* on soybean stems, but only seed coated with the antibiotic-producing isolate of *C. globosum* reduced latent colonization of *D. phaseolorum* var. *meridionalis*, and neither that isolate nor the non-antibiotic-producing one significantly decreased mortality of plants grown from seed coated with either isolate (Pereira and Dhingra 1997). However, when wheat bran colonized by either isolate of *C. globosum* was tilled into soil, the extent of colonization along the stem, latent colonization, and mortality decreased significantly. The antibiotic-producing isolate has also been shown to reduce perithecial formation and colonization of *D. phaseolorum* var. *meridionalis* on overwintering, artificially-inoculated stem pieces (Dhingra et al. 2002). *Chaetomium cupreum*, its culture filtrate, and a deproteinated extract of its culture filtrate have also been shown to be antagonistic to *P. longicolla*, although the extract has also been shown to limit soybean radicle growth during germination (Manandhar et al. 1987b; Yeh and Sinclair 1980). Various saprophytic fungi including an *Acremonium* sp., *Aspergillus terreus*, *Epicoccum purpurascens*, *Gliocladium roseum*, *Myrothecium roridum*, *Penicillium thomii*, a *Scopulariopsis* sp., and a *Stachybotrys* sp. have been shown to inhibit *D. phaseolorum* var. *sojae* growth in culture, but soil-furrowed *A. terreus*, *G. roseum*, *P. thomii*, and *T. harzianum* did not increase soybean seed emergence when the seed were inoculated with *D. phaseolorum* var. *sojae* or decrease cotyledon lesion severity, although emergence and cotyledon lesion severity were relatively high and low, respectively, when comparing the control soil-furrow or seed treatment, in this case water, with the saprophytic fungi treatments (Manandhar et al. 1987b). *Trichoderma harzianum*, *T. longibrachiatum*, *T. koningii*, and *T. virens* and, to a lesser degree, *Burkholderia glumae*, *Pseudomonas aeruginosa*, and *Serratia marcescens* have been found to inhibit radial growth of

D. phaseolorum var. *sojae* (Begum et al. 2008). No interaction was found between *Phomopsis* spp. and *Chaetomium, Paecilomyces*., *Alternaria*, *Penicillium*, *Aspergillus*, and *Cladosporium* spp. by McGee et al. (1980). No interaction was found with *D. phaseolorum* var. *sojae* and *Cercospora sojina* either (Bisht and Sinclair 1985).

One isolate of *Bacillus subtilis* and its culture filtrate extract have been found to inhibit *P. longicolla* culture growth (Cubeta et al. 1985). Additionally, prophylactic use of the *B. subtilis* isolate via spray before spraying plants with *P. longicolla* reduced pod colonization by *P. longicolla* in the greenhouse but not in the field where natural infection by *P. longicolla* was relied upon. Meanwhile, Hilty (1993) found *Bacillus pumilus* to inhibit *D. phaseolorum* var. *caulivora* in culture and to lyse almost all the spores in an incubated mixture of *B. pumilus* cells and *D. phaseolorum* var. *caulivora* spores. Additionally, the culture filtrate of *B. pumilus* inhibited spore germination and decreased germ tube length and live or autoclaved *B. pumilus* decreased disease severity on detached soybean leaflets when inoculated in conjunction with *D. phaseolorum* var. *caulivora*.

Management of Phomopsis seed decay

Fungal infestation of *Phomopsis* spp. on soybean seeds in storage increases when dried pods are machine threshed instead of hand threshed and when humidity and drying temperature are high (Sangakkara 1988). Machine threshing increases incidence of cracked seed and might facilitate *Phomopsis* spp. infection during storage. As good quality seeds for planting are sometimes scarce in the developing world, hand threshing and storage of seed in cool, dry areas might be a practical solution to the problem for small farm holders. When seed lots infected with *P. longicolla* were placed in storage, the seed infection level dropped and germination increased

(Fabrizius et al. 1997; TeKrony et al. 1982). However, DPC species have been found to survive on soybean seed stored for nine years at 0°C, with 8-10% seed moisture (Raeisi et al. 2011). Seed lots with high levels of PSD do not decrease in vigor any faster than seed lots with low levels of PSD. Seed with PSD treated with Vitavax® 200 flowable fungicide (17% carboxin + 17% thiram) prior to storage did not show any increase in vigor compared to infected untreated seed (Fabrizius et al. 1997). However, germination did increase during the first few months of storage but not during prolonged storage as compared to untreated seeds.

Potassium (K) and dolomitic lime have been found to play a role in PSD development in some areas. Soybeans grown in the field in aluminum cylinders with low K soil had significantly higher PSD incidence than soybeans grown in such soil but with added K or with added K and a sidedress of additional K (Crittenden and Svec 1974). However, when soybeans were grown in steel drums with medium K soil in the field or grown in the field without a container in either medium or high K soil, K fertilization did not have a consistent effect on PSD incidence on the cultivars grown (Andrews and Svec 1976; Svec et al. 1976). Others have found that K fertilization, but not phosphorus (P) fertilization, decreased PSD incidence on soybeans (Camper and Lutz 1977; Ito et al. 1994; Mascarenhas et al. 1976; Mascarenhas et al. 1995; Sij et al. 1985). The method of K fertilization (broadcast or furrowed) did not affect PSD (França Neto et al. 1985). Although Mascarenhas et al. (1976) found that foliar K concentration was not significantly different between different K fertilization levels even though PSD incidence declined with K fertilization, Ito et al. (1994) found that the concentration of K in leaves and soil is inversely related to PSD incidence; therefore, dolomitic lime, which decreased the concentration of K in soil and leaves, indirectly increased PSD incidence. However, Mascarenhas et al. (1995) found that dolomitic lime had no effect on PSD incidence. Sij et al.

(1985) found that K fertilization and two benomyl applications reduced seed infection more than K fertilization alone. On the other hand, Jeffers et al. (1982) found that while K fertilization, which increased foliar K concentration, consistently lowered the incidence of moldy seed, one symptom of PSD, it did not lower PSD incidence. Additionally, Spilker et al. (1981) found that soybeans grown on soil with increased P and K and given P and K foliar applications did not have a significantly different PSD incidence than soybeans grown on soil with lower levels of P and K, and Garzonio and McGee (1983) observed that soil and foliar K levels above the deficiency range did not explain the high levels of PSD found in soybeans grown in continuous soybean fields. Likewise, TeKrony et al. (1987) found no relationship between either K fertilization or seed K concentration and PSD and no relationship between either K fertilization or pod K concentration at R7 on *P. longicolla* and *D. phaseolorum* var. *sojae* pod infection at R7. However, França Neto et al. (1985) did find a significant negative correlation between both K fertilization or seed K concentration and PSD.

Other cultural practices for controlling PSD have also been studied. Camper and Lutz (1977) found that tillage had no significant effect on PSD. Grau and Oplinger (1981), however, did find that deep plowing decreased PSD compared to chisel plowing when soybean was grown in a field planted with soybean the previous year. Decreasing row width has been shown to either decrease or have no effect on PSD (Grau and Oplinger 1981).

Various organic and inorganic fungicides have been tested for their control of PSD. Zineb, maneb, captan, and tribasic copper sulfate have not been shown to be effective against PSD when applied six times in 10-day intervals from the flowering stage of soybean (Crittenden et al. 1967). However, zineb and tribasic copper sulfate reduced PSD when applied before flowering every seven days for seven weeks with the most important spraying times being those during the

flowering period. Benomyl (methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl]carbamate or Benlate 50 WP) has been shown to significantly reduce the incidence of PSD when applied at either the mid-flower or late-pod developmental stage or both and when applied at a rate of 1/8 active ingredient (a. i.) $acre^{-1}$, 1/4 a. i. $acre^{-1}$, or 1/2 a. i. $acre^{-1}$ with or without oil (Ochrex 70° viscosity) (Ellis et al. 1974). Benomyl at concentrations of 50 µg ml^{-1} have also been shown to significantly reduce incidence of PSD in seed harvested at maturity and 30 days after maturity when sprayed four times at two-week intervals 55 days after planting (Ellis and Sinclair 1976). Benomyl 50 WP + zinc + maneb 80 WP, thiophanate methyl 70 WP, benomyl 50 WP, chlorothalonil 75 WP, zinc + maneb 80 WP, and thiabendazole 98.5 WP have all been shown to significantly reduce the incidence of PSD when sprayed four times at 10-day intervals either 70 days after planting or from R2 to R6 with chlorothalonil 75 WP and thiabendazole 98.5 WP reducing PSD significantly less than the other fungicides (Prasartsee et al. 1975). Out of 13 fungicides (Afugan, Bavistin, Bavistin C-65, Benomyl, Benlate C, Benlate T, DCNA, Dithane M-45, Mertect, Polyram combi, Ronilan, Perrazole, and Zineb) tested, all fungicides had some fungistatic effect on *D. phaseolorum* var. *sojae* and all fungicides except Afugan had a fungicidal effect on *D. phaseolorum* var. *sojae* using the cellophane-transfer bioassay method (Wu and Lee 1985). Bavistin had the strongest fungistatic and fungicidal effects on *D. phaseolorum* var. *sojae*. Seed heavily infected with *D. phaseolorum* var. *sojae* and that had a low emergence rate were soaked with either Bavistin, Bavistin C-65, Benlate, Benlate C, Benlate T, Dithane M-45, Mertect, or Polyram combi dissolved in acetone or in dichloromethane, and all the fungicides showed a reduced in the percentage of *D. phaseolorum* var. *sojae*-infected stems in seedlings; however, only Bavistin and Benlate T dissolved in acetone and Bavistin C-65, Benlate, Benlate C, and Benlate T significantly improved the emergence rate of the seed. Benomyl, triphenyltin

acetate, captan, and thiram seed treatments all individually significantly reduced recovery of fungi when seed treatment was done on *P. longicolla*-infected seed with benomyl seed treatment being significantly more effective than the other seed treatments (Bolkan et al. 1976). However, triphenyltin acetate was found to be phytotoxin and reduced seed germination and emergence. Weekly applications of benomyl and a single application of benomyl at R6 have been shown to reduce PSD when harvested at R8 and at R8 and 3 weeks post-R8, respectively, although not all decreases in PSD were statistically significant (TeKrony et al. 1996). Benomyl has no significant effect on *Phomopsis* spp. seed infection when sprayed on the ground around plants rather than on the whole plants (Oh 1998). Spraying benomyl at R3 had no significant effect on PSD (Mengistu et al. 2009), although Grau and Oplinger (1981) did find a significant decrease in PSD when using this fungicide at R3. Furthermore, spraying benomyl at R7 does not give as large of a decrease in *P. longicolla* and *D. phaseolorum* var. *sojae* seed infection as R6 spraying (Lee et al. 1994). The rate at which benomyl is applied also seems to affect PSD with a rate of 0.5 lb/acre having a significantly lower reduction in PSD than a rate of 1 lb/acre when applied at R3 and R6 (Grau and Oplinger 1981).

Resistance to Phomopsis seed decay

A number of plant introductions (PIs), breeding lines, and cultivars are thought to be resistant to PSD. ‘Lee’, ‘Ogden’, ‘Hill’, ‘Dorman’, ‘Hood’, ‘Dare’, ‘Delmar’ (Crittenden et al. 1967), PI 80837 (Yelen and Crittenden 1967); PI 82264 (Walters and Caviness 1973), PI 181550[1] (Athow 1973), PI 204331, PI 205907, PI 205908, PI 205912, PI 219653, PI 259539, PI 279088, PI 341349 (Paschal and Ellis 1978), TGm 685, TGm 686 (Ndimande et al. 1981), OX-303

[1] Athow (1973) originally stated that PI 161550 was the resistant PI; however, as this soybean plant introduction never existed (R. Nelson, personal communication), it seems that Brown et al. (1987) correctly fixed this typo to PI 181550.

(TeKrony et al. 1984), PI 417274, PI 416921, PI 417303, PI 417460, PI 404169A, PI 417046, PI 416946 (Ploper and Abney 1985), 'Gnome' (Anderson and Buzzell 1985), 'Arksoy', PI 200510, PI 209908 (Ross 1986), 'Ransom' (Athow 1987), PI 417479, PI 360841 (Brown et al. 1987), PI 594778, PI 594712, 594603A (Mengistu et al. 2010), PI 424324B, PI 458130 (Li et al. 2011) and 'Taekwangkong' (Sun et al. 2012a) have all been found to have resistance to PSD. Additionally, 63 PIs of MG II, III, and IV have been found to be resistant to PSD in a germplasm screening experiment (Smith et al. 2008a). Further, lines derived from the Brazilian cultivar Santa Maria, PI 227687, and PI 229358 were shown to have resistance to PSD (Berger and Hinson 1984). The NCPR83 family of lines, whose lineage includes 'Arksoy', PI 200510, and PI 209908, has also been found to be resistant to PSD (Ross 1986). The PSD-resistant line MO/PSD-0259 (PI 562694) was developed from a cross between PI 417479 and Merschman 'Dallas' (Minor et al. 1993). The PSD-resistant lines SS93-6012 and SS93-6181 were developed from MO/PSD-0259 and 'Asgrow 3834' (Pathan et al. 2009). Mengistu et al. (2010) found Arksoy, MO/PSD-0259, and NCPR83-47 susceptible to PSD and PI 200510, PI 82264, PI 417479, and PI 360841 moderately susceptible to PSD under irrigation and delayed harvest using the Phomopsis/percent seed infection index (PSII), which divides the percent seed infection of an accession by the percent seed infection of a susceptible standard with a similar maturity group. Also based on the PSII, DP 3478 and R01-769F were found to be resistant and 'Croton,' DG 4460, PI 587585B, R01-416F, and R01-581F were found to be moderately resistant (Mengistu et al. 2012). It should be noted that the PSII is an arbitrary system of classification that seeks to accomplish what statistical methodologies can do better. Breeding for PSD resistance is possible because of broad-sense heritabilities between 0.44 and 0.83 and narrow-sense heritabilities between 0.42 and 0.49 (Anderson and Buzzell 1985; Zimmerman and Minor 1993).

PI 417479 has been shown to confer resistance to PSD by two complementary dominant nuclear genes (Zimmerman and Minor 1993). Reciprocal crosses indicated that there was no maternal effect on resistance. Resistance to PSD in MO/PSD-0259 and PI 80837 is controlled by only one dominant nuclear gene, but based on the F_2 population of the cross PI 80837 × MO/PSD-0259, the two genes were different (Jackson et al. 2005). One of the resistance genes from PI 417479 was apparently lost during development of MO/PSD-0259 (Smith et al. 2008b). Two dominant complementary genes have been shown to confer PSD resistance in PI 360841 (Smith et al. 2008b). It is very likely that PI 360841 and PI 80837 share a resistance gene. A study of the F_2 population of cross MO/PSD-0259 × PI 360841 has shown that the two complementary dominant genes from PI 360841 are different from the single dominant gene in MO/PSD-0259, which was derived from PI 417479 (Smith et al. 2008b). It is not yet known whether the resistance genes from PI 360841 and PI 417479 are at the same locus (Smith et al. 2008b).

Restriction fragment length polymorphism (RFLP) markers were the first markers used to elucidate the genomic locations of PSD resistance genes. RFLP marker A708 located on linkage group (LG) F accounted for 23.2% and 62.2% of the phenotypic variation in PSD for the cross PI 417479 × 'AP 350' in 1993 and 1994, respectively, and 21.7% and 21.0% of the phenotypic variation in PSD for the cross PI 417479 × 'Williams 82' in 1993 and 1994, respectively (Berger and Minor 1999). RFLP marker A162 located on LG H accounted for another 4.5% of the phenotypic variation in PSD for the cross PI 417479 × AP 350 in 1994. Amplified fragment length polymorphism (AFLP) markers AF1, AF2, and AF3 were also associated with PSD resistance in the BC_1F_2 of 'Hwaeomputkong' × PI 417479 (Yun et al. 2007). Simple sequence repeat (SSR) markers were another type of marker used for linkage analysis and were used to map the proposed resistance genes *Rpsd1* and *Rpsd2* of PI 80837 and MO/PSD-0259,

respectively (Jackson et al. 2009). *Rspd1*, located on LG B2, is 4.3 cM from SSR marker Sat_177 and 15.8 cM from SSR marker Sat_342. *Rspd2*, located on LG F, is 5.9 cM from SSR marker Sat_317 and 12.7 cM from SSR marker Sat_120. *Rspd2* is approximately 20.9 cM from RFLP marker A708, which might explain the small phenotypic variation in PSD accounted for by A708. SSR markers were also used to map the resistance quantitative trait loci (QTLs) *PSD 6-1* and *PSD 10-2* of Taekwangkong and SS2-2, a susceptible supernodulating mutant (Sun et al. 2012a), respectively (Sun et al. 2013). *PSD 6-1* and *PSD 10-2* accounted for 46.3% and 14.1% of the phenotypic variation in PSD, respectively. *PSD 6-1* is located between Satt100 and Satt460 on LG C2 and *PSD 10-2* is located between Sat_038 and Satt243 on LG O. Both of these QTLs are associated with maturity QTLs located at almost the same position on the LGs. SSR marker Sct_033 located on LG F has also been associated with PSD resistance in the BC_1F_2 of 'Hwaeomputkong' × PI 417479 (Yun et al. 2007).

Chapter 2: Characterization of Morphologically Atypical *Diaporthe sojae* Isolates and Implications for the Taxonomy of Fungi in the *Diaporthe*/*Phomopsis* Complex

Introduction

Phomopsis longicolla Hobbs was named to account for the morphologically novel fungus that was predominately found to cause Phomopsis seed decay (PSD) on soybean (*Glycine max* (L.) Merr.) (Hobbs et al. 1985; Kmetz et al. 1974; Kmetz et al. 1978). *P. longicolla* and *Diaporthe phaseolorum* var. *sojae* (Lehman) Wehm., the fungus that previously was thought to cause both pod and stem blight and seed decay, differed in stroma morphology, pycnidial beak size, mean alpha-conidia length, width and length/width (L/W) ratio, beta-conidia proportion, conidiophore morphology, and presence of a teleomorph when cultures grown on potato dextrose agar (PDA) were examined (Hobbs et al. 1985). *P. longicolla* had massive, effuse stromata, longer pycnidial beaks, smaller mean alpha-conidia length and L/W ratio and larger mean alpha-conidia width, seldom beta-conidia formation, more frequent branching of conidiophores, and no teleomorph. However, the morphologies of these two species have been shown to overlap. *P. longicolla* isolates have been found producing pulvinate stromata and *D. phaseolorum* var. *sojae* isolates have been found forming effuse stromata (Chao and Glawe 1984; Pioli et al. 2003; Zhang et al. 1998). Luttrell (1947) and Nevena et al. (1997) found long pycnidial beaks produced by *D. phaseolorum* var. *sojae* isolates. Mean alpha-conidia length and width of a *Phomopsis* sp. and of *D. phaseolorum* var. *sojae* on acidified PDA (APDA) were found by Kmetz (1975) to be similar, i.e., 6.9 × 2.4 µm and 6.8 × 2.4 µm, respectively, and alpha-conidia length, width, and L/W ratio of *P. longicolla* isolates were found by Vidić et al. (2013) to be both greater and less than those found by Hobbs et al. (1985). The proportion of beta-conidia in cultures *of P. longicolla* and *D. phaseolorum* var. *sojae* can be influenced by the environment. *P. longicolla* isolates normally producing alpha-conidia have been reported to produce beta-conidia, some even exclusively,

when exposed to low temperatures for a week, but this was not a characteristic shared by all isolates. One *P. longicolla* isolate only formed alpha-conidia regardless of low temperature exposure (Vidić et al. 2013). Subculturing can also have an effect on the proportion of beta-conidia produced by a culture as Kmetz (1975) found that subculturing *D. phaseolorum* var. *sojae* increased the frequency of beta-conidia, although this characteristic was not seen in *P. longicolla*. Branching of conidiophores of *D. phaseolorum* var. *sojae*, from which multiple conidiogenous cells develop, has been found to be common (Jensen 1983). A *P. longicolla* isolate has been reported to form fertile perithecia, albeit rarely and sparingly, on autoclaved elm bark, with morphological characteristics, viz., ascus and ascospore length and width, that put it in a canonical discrimination analysis-derived grouping with isolates of only *D. phaseolorum* var. *sojae* (Fernández and Hanlin 1996), and another isolate of *P. longicolla* has been observed to form perithecia on soybean stems, but single-ascospore isolates could not be obtained from them (Vidić et al. 2013). *D. phaseolorum* var. *sojae* has the ability to form perithecia, which subculturing can suppress in certain isolates, but some isolates only produce pycnidia on PDA and soybean tissue (Athow and Caldwell 1954; Fernández and Hanlin 1996; Gerdemann 1954; Hildebrand 1954; Hildebrand 1956; Jaccoud-Filho et al. 1997; Kmetz 1975; Kurata 1960; Luttrell 1947; Pioli et al. 2003; Zhang et al. 1998). Therefore, the lack of perithecial formation cannot be used to discriminate *P. longicolla* from *D. phaseolorum* var. *sojae*. Additionally, *P. longicolla* has been found to cause pod and stem blight, a disease usually attributed to *D. phaseolorum* var. *sojae*, and the two fungi can be highly or moderately aggressive in causing PSD (Cui et al. 2009; Kmetz 1975; Kmetz et al. 1979; Vidić et al. 1998).

In addition to *P. longicolla* and *D. phaseolorum* var. *sojae* being morphologically indistinguishable, there also appear to be no definitive morphological or pathological methods

that can be used to discriminate them from the other two members of the *Diaporthe*/*Phomopsis* complex (DPC). Those members are *Diaporthe phaseolorum* var. *caulivora* Athow & Caldwell and *Diaporthe phaseolorum* var. *meridionalis* F.A. Fernández, the causal organisms of northern and southern stem canker, respectively. Between *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis*, perithecial length, arrangement, and color, ascus and ascospore lengths, stroma morphology, and mycelial color of both the front and reverse on PDA and mycelial texture have been found to overlap (Athow and Caldwell 1954; Chao and Glawe 1984; Chao and Glawe 1985; Fernández and Hanlin 1996; Kmetz 1975; Lehman 1923; Luttrell 1947; Nevena et al. 1997; Pioli et al. 2003; Sato et al. 1993; Vrandecic et al. 2005). Moreover, isolates of *D. phaseolorum* var. *caulivora* have been found at times to not produce perithecia on PDA or to lose this ability after being subcultured or isolated on PDA (Fernández and Hanlin 1996; Kmetz 1975; Kulik 1985). An isolate of *D. phaseolorum* var. *sojae* and an isolate of *P. longicolla* have been shown to cause stem canker in 78- to 85-day-old soybean plants, although the fungi were isolated some distance away from the point of inoculation (Kmetz et al. 1979). Thus, using stem canker symptoms to distinguish *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis P. longicolla* and *D. phaseolorum* var. *sojae* from might not be feasible.

Due to the labyrinthine morphology of fungi in the DPC, efforts to elucidate the phylogeny of these fungi have been attempted using molecular tools. Zhang et al. (1998) used maximum parsimony and maximum likelihood methods on internal transcribed spacer (ITS) regions sequenced with ITS1 and ITS2 primers of various fungal accessions in the DPC. They found that while *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* formed one clade each, with bootstrap proportions greater than 70, *D. phaseolorum* var. *sojae* formed two groups

with bootstrap proportions of 69 and 100 of which the former contained the only unique sequence of *P. longicolla* included in the analysis, and two other groups with bootstrap proportions less than 69 (60 and <50). Bootstrap proportions greater than 70 have been found to provide accurate estimates of the phylogeny of simulated taxa (Hillis and Bull 1993). A group containing *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* had a bootstrap proportion of only 60. Meanwhile, random amplified polymorphic DNA (RAPD) analysis suggests that *D. phaseolorum* var. *sojae* is a polyphyletic taxon genetically similar to both *P. longicolla* and *D. phaseolorum* var. *caulivora*, but isolates of *D. phaseolorum* var. *sojae* genetically distinct from either of those two species exist (Fernández and Hanlin 1996). Using a combination of five genomic loci and Bayesian analysis, Gomes et al. (2013) found that *D. phaseolorum* var. *sojae* had two well-supported clades, one containing *P. longicolla* and another containing *Diaporthe melonis*, while *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* formed distinct clades. Therefore, *P. longicolla* and *D. phaseolorum* var. *sojae* were set as synonyms and the name given to the polyphyletic taxon was *Diaporthe sojae* Lehman.

The objective of this study was to identify and characterize an atypical isolate of a fungus belonging to the *Diaporthe*/*Phomopsis* complex.

Materials and Methods

Isolation and morphological identification of a fungal isolate causing PSD

Soybean seeds from plants grown in Urbana, IL in 2012 were found to be enveloped with white mycelia, a sign typical of PSD. These seeds were surface disinfected in 95% ethanol for 30 seconds, followed by 0.5% sodium hypochlorite for 3 min, and finally by rinsing them twice in

sterile double-distilled water. Seeds were then placed on acidified potato dextrose agar (APDA) prepared from Difco™ PDA (Becton, Dickinson and Company, Franklin Lakes, NJ) amended with 800 µL of 85% lactic acid (Thermo Fisher Scientific Inc., Waltham, MA) per liter to lower the pH of the agar to 4.5. The cultured seeds were incubated in an Adaptis A350 growth chamber with a CMP6010 control system (Conviron, Winnipeg, Canada). The temperature was maintained at 22 ± 1°C, the relative humidity was maintained at 70 ± 5%, the average light intensity was maintained at 75 µmol m^{-2} s^{-1}, and the light period was set to consecutive 12 h light and 12 h dark intervals. Light intensity was measured by putting a MQ-200 Quantum Meter (Apogee Instruments, Inc., Logan, UT) on top of a Petri dish in three different locations on each of the three shelves and taking the average of the readings. A fungus that was identified as a member of the DPC based on its morphological characteristics was subcultured on APDA, and a single-alpha-conidial isolate was obtained by placing pycnidia of the fungus in a test tube with sterile double-distilled water, vortexing the test tube, spreading the resulting suspension on an APDA plate using a bacterial loop, and using a 1 mL NORM-JECT® Luer lock syringe (Henke-Sass, Wolf GmbH, Tuttlingen, Germany) with an 18Gx1 (1.2 mm x 25 mm) PrecisionGlide™ needle (Becton, Dickinson and Company, Franklin Lakes, NJ) to pick up a single-alpha-conidium with the syringe tip and place it on a clean APDA plate with circles made with a marker to signify where the syringe tip should be moved to displace the conidia for later confirmation of isolation success. The syringe tip was flame-sterilized after each single-alpha-conidial isolation attempt. Mycelial growth similar to members of the DPC within the circles signified a successful isolation. This isolate, designated IL12-Ds-1, was morphologically characterized on APDA and autoclaved soybean stem and leaf pieces. Autoclaving was conducted for 30 minutes at 121°C. In order to mimic overwintering and to attempt to induce the

formation of beta-conidia, IL12-Ds-1 cultures that had developed pycnidia on APDA were stored for seven days at 4°C and -20°C, and conidia type was analyzed 14 days after the cultures were returned to the growth chamber (Vidić et al. 2013).

After two generations of subculturing IL12-Ds-1, a single-alpha-conidial isolation was performed on IL12-Ds-1, and a fungus with a morphology novel to the DPC was obtained. This isolate, designated IL12-Ds-2, was morphologically characterized on APDA and on autoclaved soybean leaf pieces, stem pieces, pods, and seed on water agar, and its conidial dimensions were measured from pycnidia on APDA and from pycnidia that had developed on autoclaved soybean leaf pieces cultured on water agar. Single-conidial isolations and temporally successive subculturing were attempted with IL12-Ds-2 to determine if isolates with IL12-Ds-1 morphology would develop. After six generations of subculturing IL12-Ds-1, one culture out of 50 had stroma that lacked pycnidia encompassing three-quarters of the Petri dish. In order to produce a single-spore isolate from a culture that lacked pycnidia, a stromatal plug from the culture was placed on water agar with autoclaved soybean leaf pieces. Once pycnidia formed on leaves, those leaves were placed in a test tube with sterile double-distilled water, the test tube was vortexed, and the conidial suspension was spread onto an APDA plate using a bacterial loop. The rest of the isolation protocol was identical to the procedure described above. The resulting isolate, designated IL12-Ds-3, was then morphologically characterized on APDA.

After four and seven generations of subculturing, the morphological characteristics of IL12-Ds-1 changed and were subsequently characterized on APDA. Pycnidial beaks, which formed more readily after seven generations of subculturing, and alpha and beta-conidia were photographed using an Olympus DP70 digital camera (Olympus America Inc., Center Valley, PA) attached to an Olympus SZX12 stereo microscope and an Olympus BX51 microscope using a phase-contrast

lens, respectively, and their dimensions were measured using ImageJ 1.47 (Rasband 1997-2014). Conidial dimensions from pycnidia on autoclaved soybean leaf pieces cultured on water agar were examined as well. Single-conidial isolations were attempted on beta-conidia of IL12-Ds-1. The time from the initial isolation of IL12-Ds-1 and its seventh generation of subculturing was about one year. For analysis of conidia, one-month-old seventh-generation cultures of IL12-Ds-1 incubated either in the growth chamber or in a closed translucent plastic storage container with a natural photoperiod that was kept on a shelf in the laboratory were flooded with sterile double-distilled water, and the pycnidia were dislodged using a sterile glass rod. A hemocytometer was subsequently used to determine the proportions of alpha and beta-conidia from each culture. In order to see if changes in conidial development were due to increased depletion of resources, IL12-Ds-1 cultures were made by placing a 4 mm mycelial plug of IL12-Ds-1 cut with a cork borer from the edge of a growing six-day old culture of IL12-Ds-1 mycelial-side down in the center of either an APDA or water agar plate. The plates were laid out in a completely randomized design with five replications of both media in either the growth chamber or a closed plastic container. Mycelial growth was measured as the average of the longest length of mycelial growth observed from the reverse and its perpendicular. Mycelial growth was measured for five days. PROC MIXED in SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC) was used to test the effects of incubation area and media and their interaction on mycelial growth with mycelial growth data treated as repeated measures. The covariance structure used was AR(1), and the degrees of freedom were corrected with the Kenward-Roger approximation. A p-value of 0.05 was used to determine statistical significance. In an attempt to induce a reversion of the morphological changes that occurred, a mycelial plug of the non-subcultured single-alpha-conidial isolate of IL12-Ds-1 was plated on a water agar plate containing autoclaved soybean

leaves, and a single-alpha-conidial isolation was made from conidia from the pycnidia that formed on the leaves. The morphological characteristics of this isolate were subsequently characterized on APDA.

Identification of the IL12-Ds-1 and IL12-Ds-2 by ITS sequencing

A seventh generation subculture of IL12-Ds-1 and an isolate from the non-subcultured IL12-Ds-1 previously described were grown on Difco™ Potato Dextrose Broth (Becton, Dickinson and Company, Franklin Lakes, NJ) and IL12-Ds-2 was grown on APDA. From these cultures, genomic DNA was extracted using FastDNA™ SPIN Kits (MP Biomedicals, Santa Ana, CA). Amplification with the polymerase chain reaction (PCR) was performed on a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using 1.5 mM 10X PCR buffer with 15mM $MgCl_2$, 0.1 mM dNTPs, 0.1 µM of both ITS1 and ITS4 primers (White et al. 1990), 1 unit Taq (Bioline Reagents Ltd, London, UK), and 200 ng μl^{-1} DNA with a total volume of 25 µl per reaction. The PCR settings were 94°C for 5 min, 35 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min. PCR products were purified using QIAquick® PCR Purification Kits (Qiagen, Venlo, The Netherlands) and was then sent to the W.M. Keck Center for Comparative and Functional Genomics' High-Throughput Sequencing and Genotyping Unit for fragment analysis on a 3730xl DNA Analyzer (Life Technologies Corporation, Carlsbad, CA). Sequences were created from raw nucleotide data using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI), which included trimming the outer portions of the sequences containing low quality data and manually ascertaining the identity of nucleotides found to be ambiguous by the program. The sequenced ITS regions were then aligned against the sequences in the National Center for Biotechnology Information's (NCBI) GenBank® using NCBI's Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990; Benson et al. 2008). Additionally, a

phylogenetic tree was created with the DNA sequences of all the *P. longicolla*, *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* isolates that had been deposited in GenBank in the Zhang et al. (1998) paper. The tree was created after multiple sequence alignment with Clustal Omega on the European Bioinformatics Institute (EMBL-EBI) web server using the maximum likelihood method with bootstrapping (1000 replications) on the MEGA6 software package with *Gnomonia gnomon* (GenBank accession AY818957) as the outgroup (McWilliam et al. 2013; Sievers et al. 2011; Tamura et al. 2013). The phylogenetic tree was visualized using FigTree 1.4 (Rambaut 2012).

Results

Before extensive subculturing, on APDA, IL12-Ds-1 had white, floccose mycelia that sometimes turned green, especially at a young age, and that covered the plate in 7 to 10 d. The reverse color of IL12-Ds-1 on APDA was light to dark brown. Stromata were pulvinate and contained mutic pycnidia in which alpha-conidia solely formed on conidiogenous cells developed from simple conidiophores that were rarely branched. However, some beaks were observed, albeit very rarely (Figure 2.1). Perithecia were never detected on APDA or soybean stems or leaves. Storing the APDA plates at 4°C or -20°C for seven days did not change the conidia formed inside pycnidia, i.e., beta-conidia never developed in the pycnidia of these cultures.

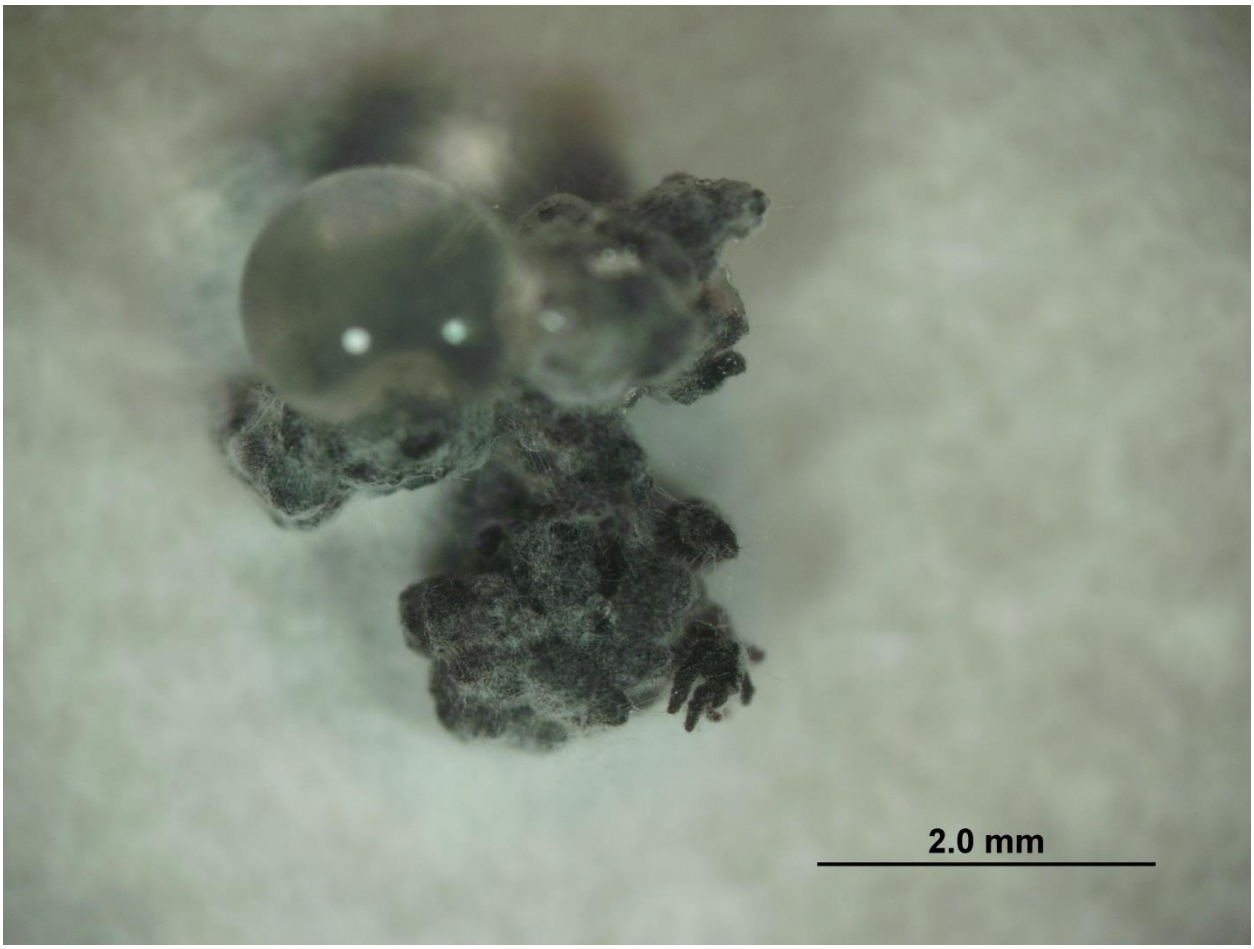

Figure 2.1. IL12-Ds-1 isolate with mutic pycnidia and pycnidia with long beaks on APDA.

After four generations of subculturing, stroma of IL12-Ds-1 became effuse, and after seven generations of subculturing, pycnidia frequently developed beaks, and beta-conidia were found inside pycnidia, in addition to alpha-conidia. The conidiophores were still simple and rarely branched, and mycelial color and morphology remained the same. Out of 54 pycnidial beaks measured, the mean length was 331 µm, and the range was 107-689 µm. Out of 100 alpha conidia and 50 beta-conidia obtained from pycnidia developed on APDA, the mean dimensions for the alpha-conidia were 6.3 × 2.8 µm, with a range of 4.5-8.0 × 1.8-4.6 µm, while the mean dimensions for the beta-conidia were 23.9 × 1.8 µm, with a range of 19.4-28.0 × 1.3-2.4 µm. All

attempts at single-beta-conidial isolations failed. Out of 100 alpha-conidia obtained from pycnidia developed on soybean leaves, the mean dimensions for the alpha-conidia were 6.4 × 2.7 µm, with a range of 4.7-8.2 × 1.9-3.5 µm. Although no beta-conidia formed, an intermediate form of conidia between alpha and beta-conidia developed. These had mean dimensions of 9.3 × 2.2 µm, with a range of 7.8-10.7 × 1.9-2.9 µm based on 14 intermediate conidia (Figure 2.2). These intermediate conidia were not biguttulate like the alpha-conidia but rather multiguttulate. The proportions of alpha and beta-conidia in IL12-Ds-1 APDA cultures showed considerable variability across the 25 growth chamber-incubated cultures. The percentage of alpha-conidia ranged from 36% to 98%, with a mean of 80%, and the percentage of beta-conidia ranged from 2% to 64%, with a mean of 20%. IL12-Ds-1 cultures had significantly greater mycelial growth when left on a laboratory shelf in a translucent plastic container than when incubated in the growth chamber, but the effect of media and the interaction between media and incubation area were not significant. The IL12-Ds-1 cultures incubated in a plastic container on a laboratory shelf also failed to produce beta-conidia but were otherwise similar to the IL12-Ds-1 cultures in the growth chamber. Pycnidia of IL12-Ds-1 incubated in a plastic container on a laboratory shelf lacked beta-conidia and contained only alpha-conidia. The single-alpha-conidial isolate from the non-subcultured isolate of IL12-Ds-1 had effuse stroma and beaked pycnidia but did not have beta-conidia present in its pycnidia.

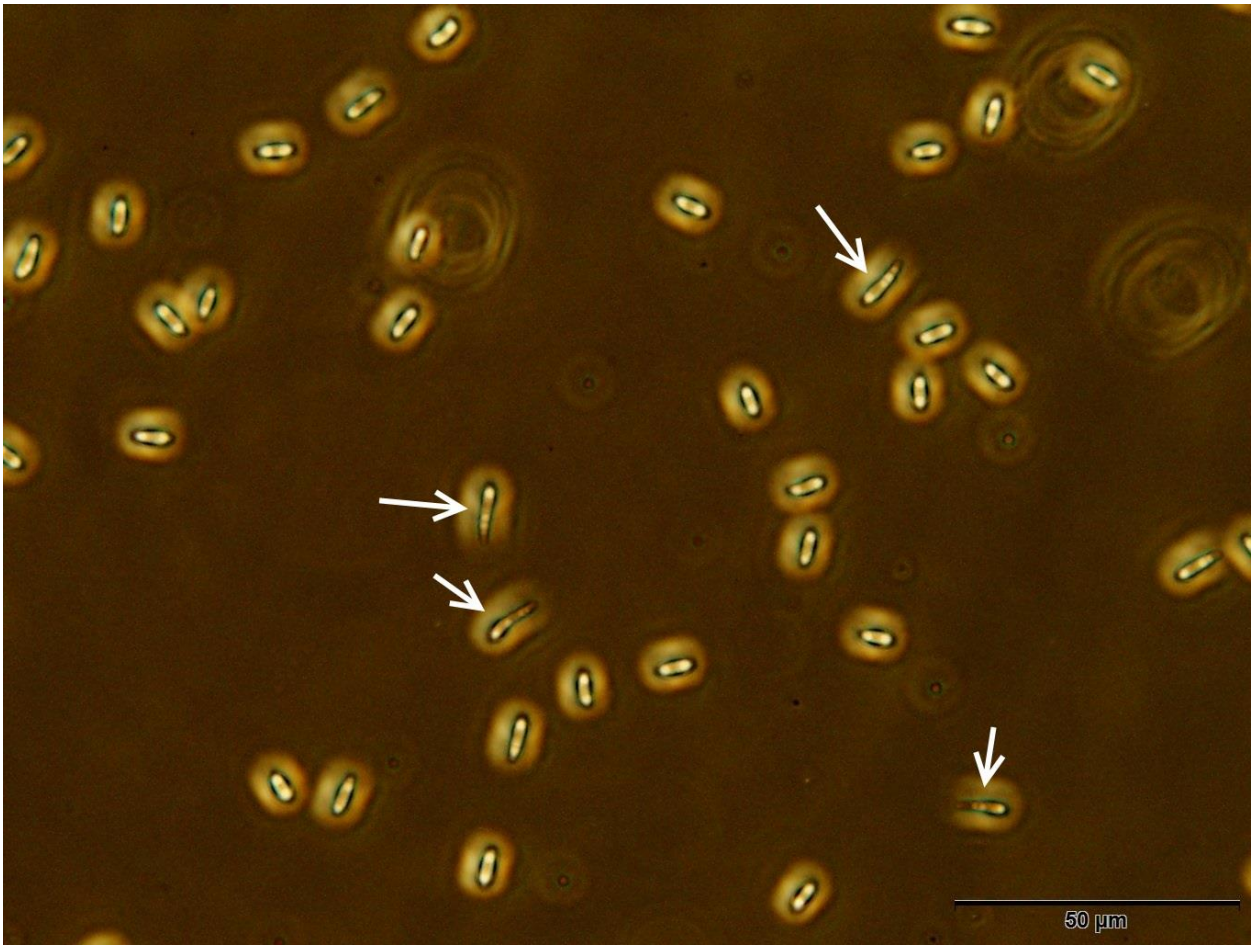

Figure 2.2. Alpha-conidia and conidia intermediate between alpha and beta-conidia (arrows) from pycnidia of IL12-Ds-1 on soybean leaves.

On APDA, IL12-Ds-2 produced abundant oozing mutic pycnidia and inconspicuous white mycelia, and colonies had a black reverse color (Figure 2.3-2.5). IL12-Ds-2 pycnidial development was also very abundant on soybean leaves, stems, pods, and seed (Figure 2.6-2.9). Perithecial development was never observed on any substrate. IL12-Ds-2 was observed forming pycnidia on soybean stem pubescence (Figure 2.10). Mycelial growth of IL12-Ds-2 was notably slower than that of IL12-Ds-1. Out of 100 alpha-conidia obtained from pycnidia that developed on APDA, the mean dimensions were 6.8 × 3.0 µm, with a range of 4.8-9.3 × 2.2-4.3 µm. Out of 100 alpha-conidia obtained from pycnidia that developed on soybean leaves, the mean

dimensions were 6.5 × 2.9 µm, with a range of 4.8-8.1 × 1.9-3.7 µm. Alpha-conidia were the only type of conidia produced by IL12-Ds-2 on either medium. Neither single-alpha-conidial isolates nor temporally successive subcultures produced morphologically different isolates. On APDA, IL12-Ds-3 had a pink reverse and was entirely composed of stroma (Figure 2.11-2.12). The isolate was also devoid of pycnidia.

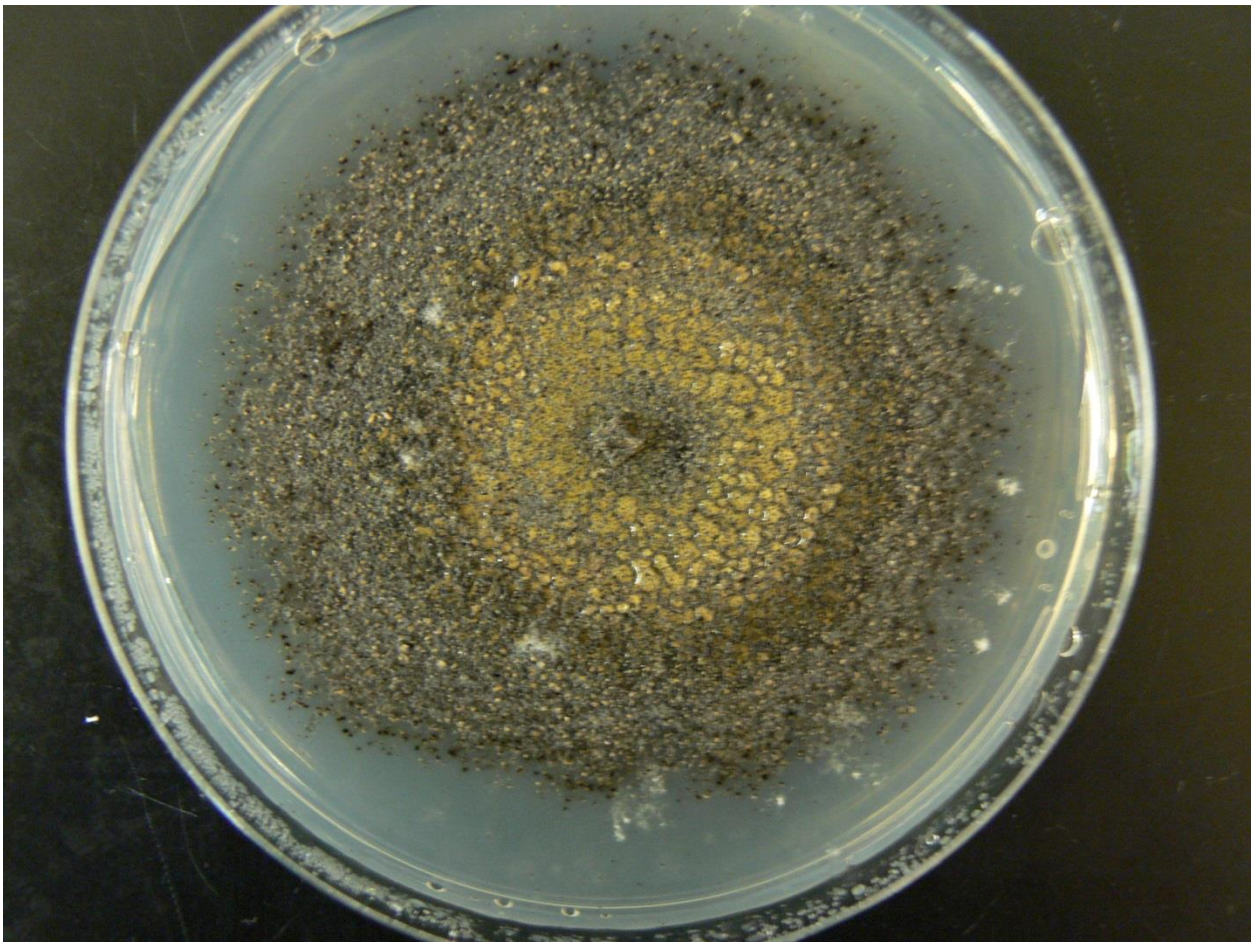

Figure 2.3. Front view of IL12-Ds-2 on APDA.

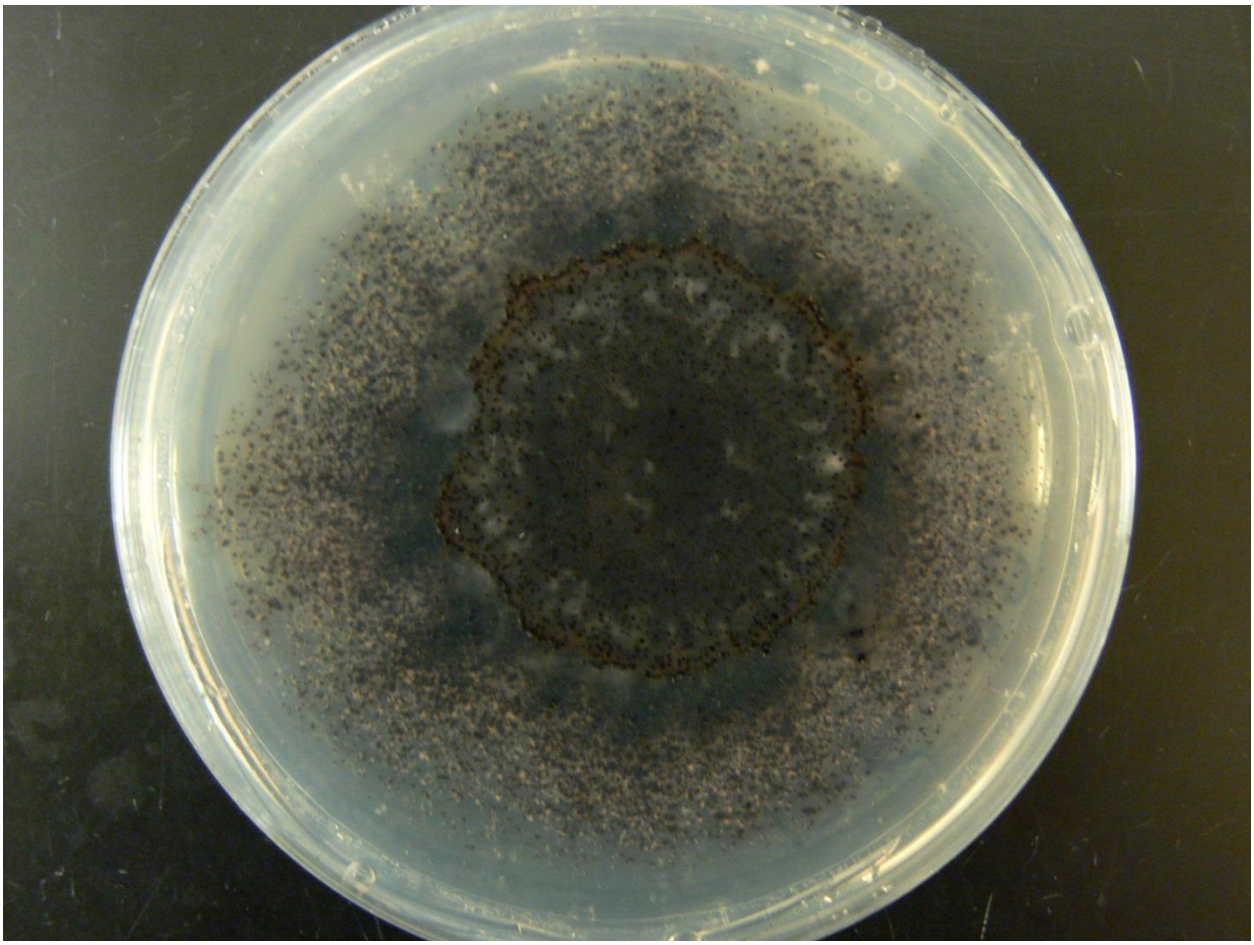

Figure 2.4. Reverse view of IL12-Ds-2 on APDA.

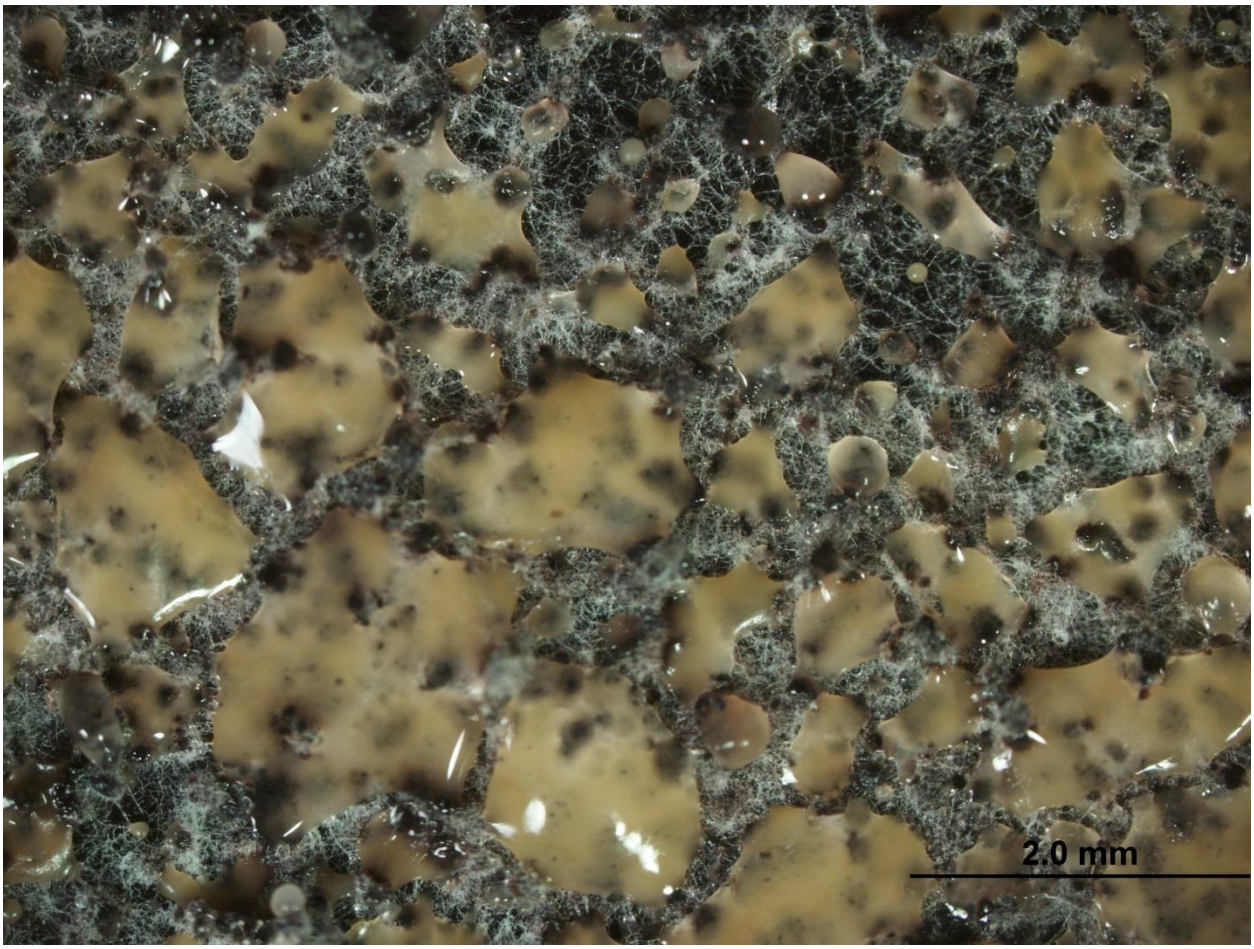

Figure 2.5. Oozing pycnidia of IL12-Ds-2 on APDA.

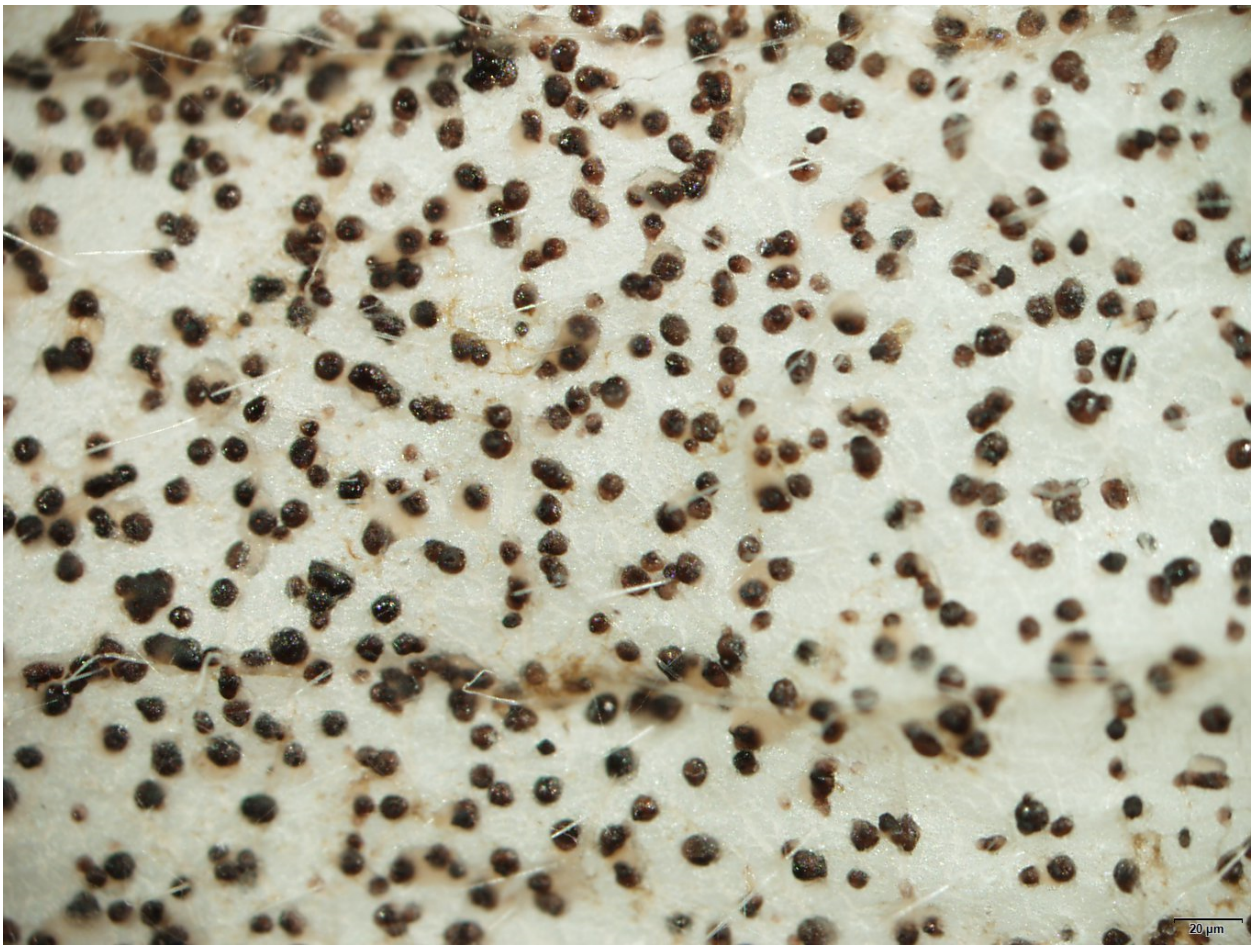

Figure 2.6. Pycnidia of IL12-Ds-2 on a soybean leaf plated on water agar.

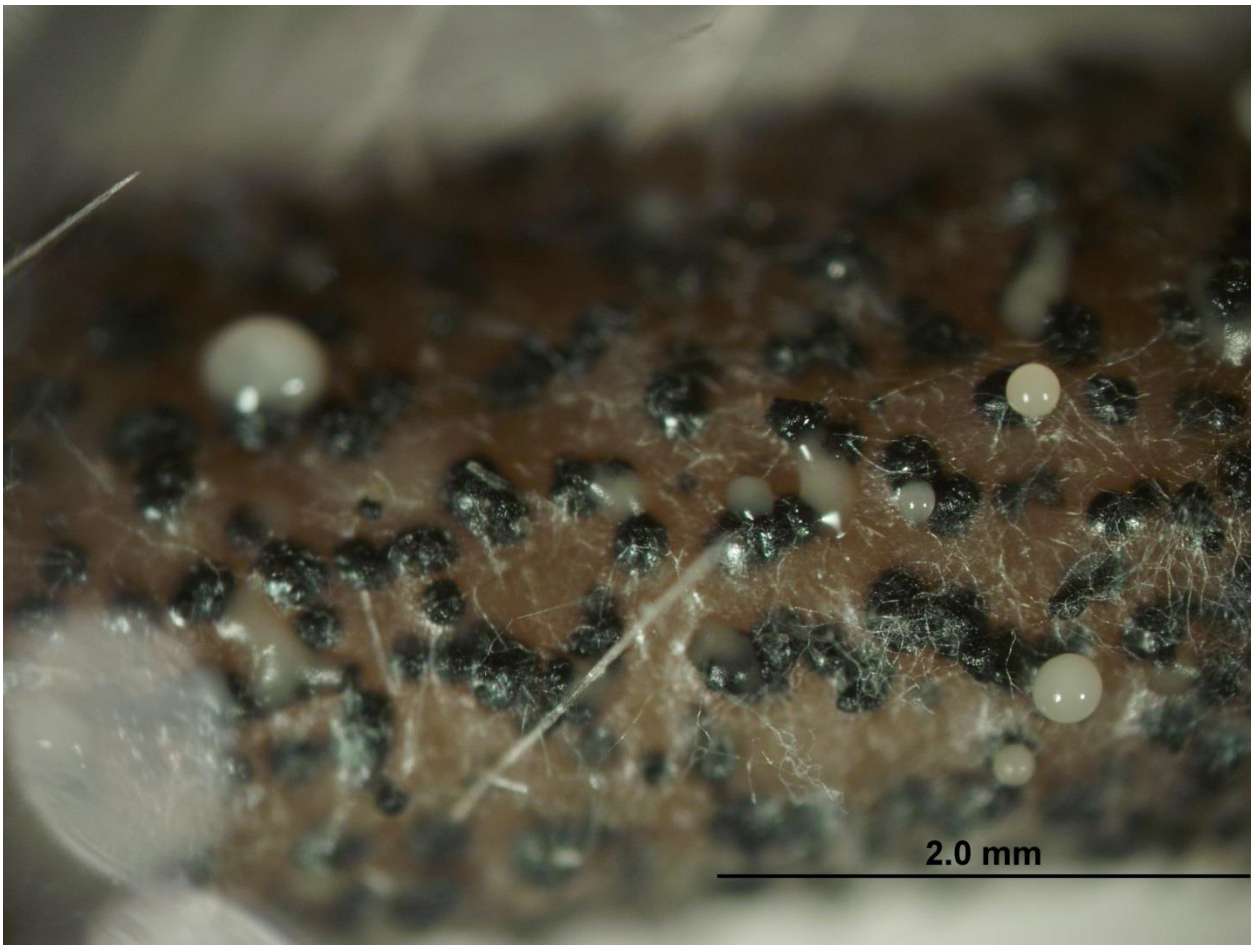

Figure 2.7. Pycnidia of IL12-Ds-2 with conidial exudates on a soybean stem plated on water agar.

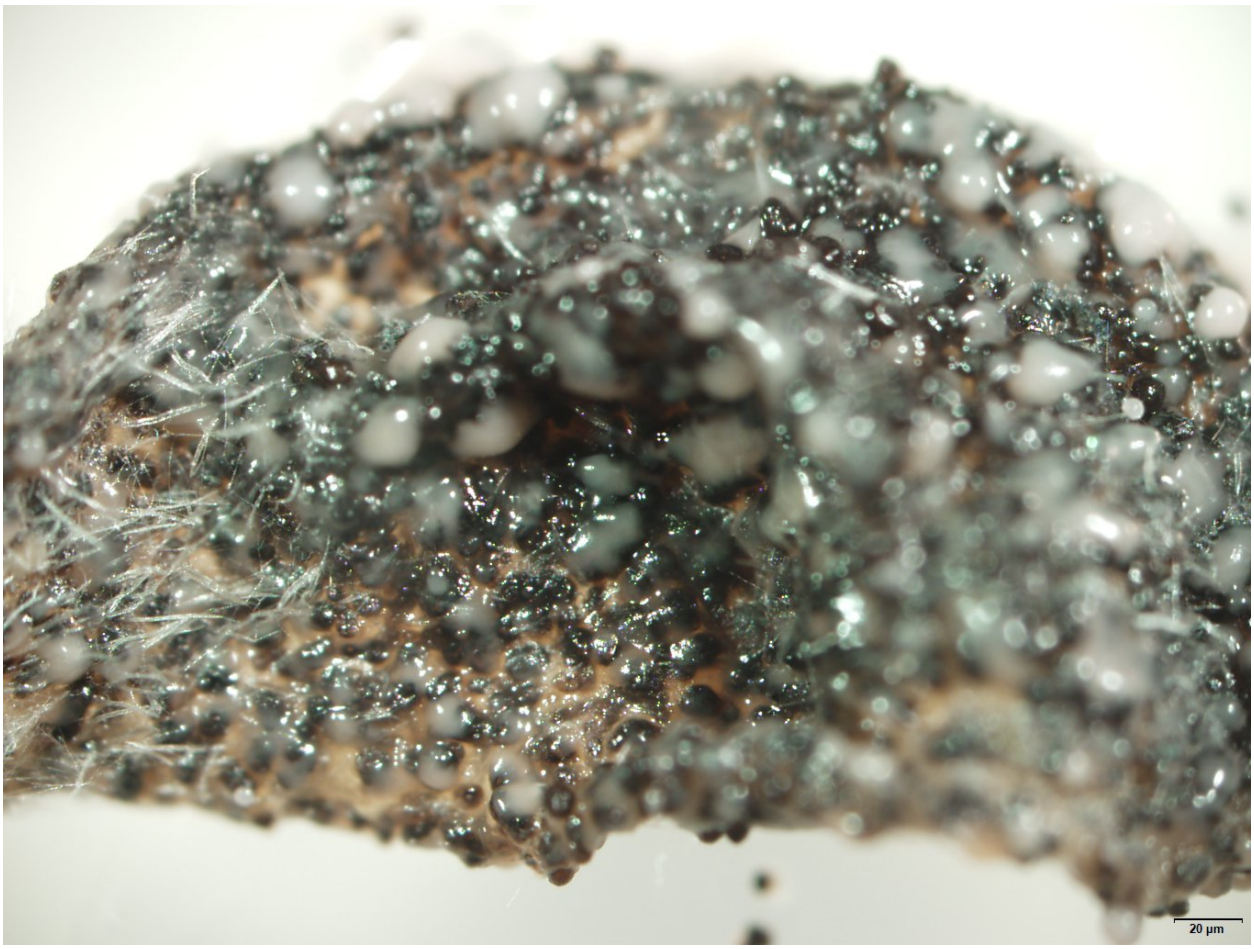

Figure 2.8. Pycnidia of IL12-Ds-2 on a soybean pod with no seed present plated on water agar.

Figure 2.9. Pycnidia of IL12-Ds-2 with conidial exudates on a soybean seed plated on water agar.

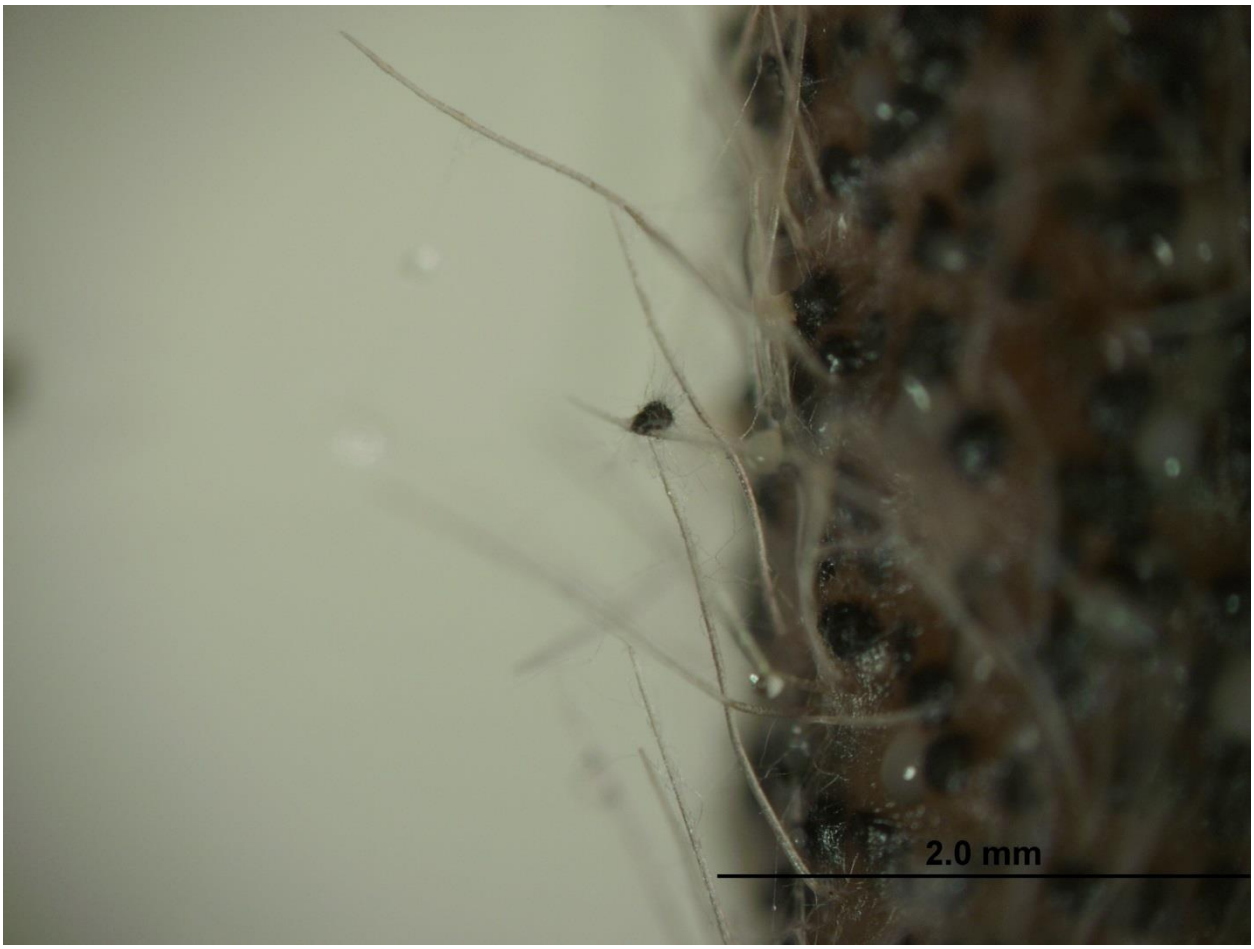

Figure 2.10. A pycnidium of IL12-Ds-2 growing on the pubescence of a soybean stem plated on water agar.

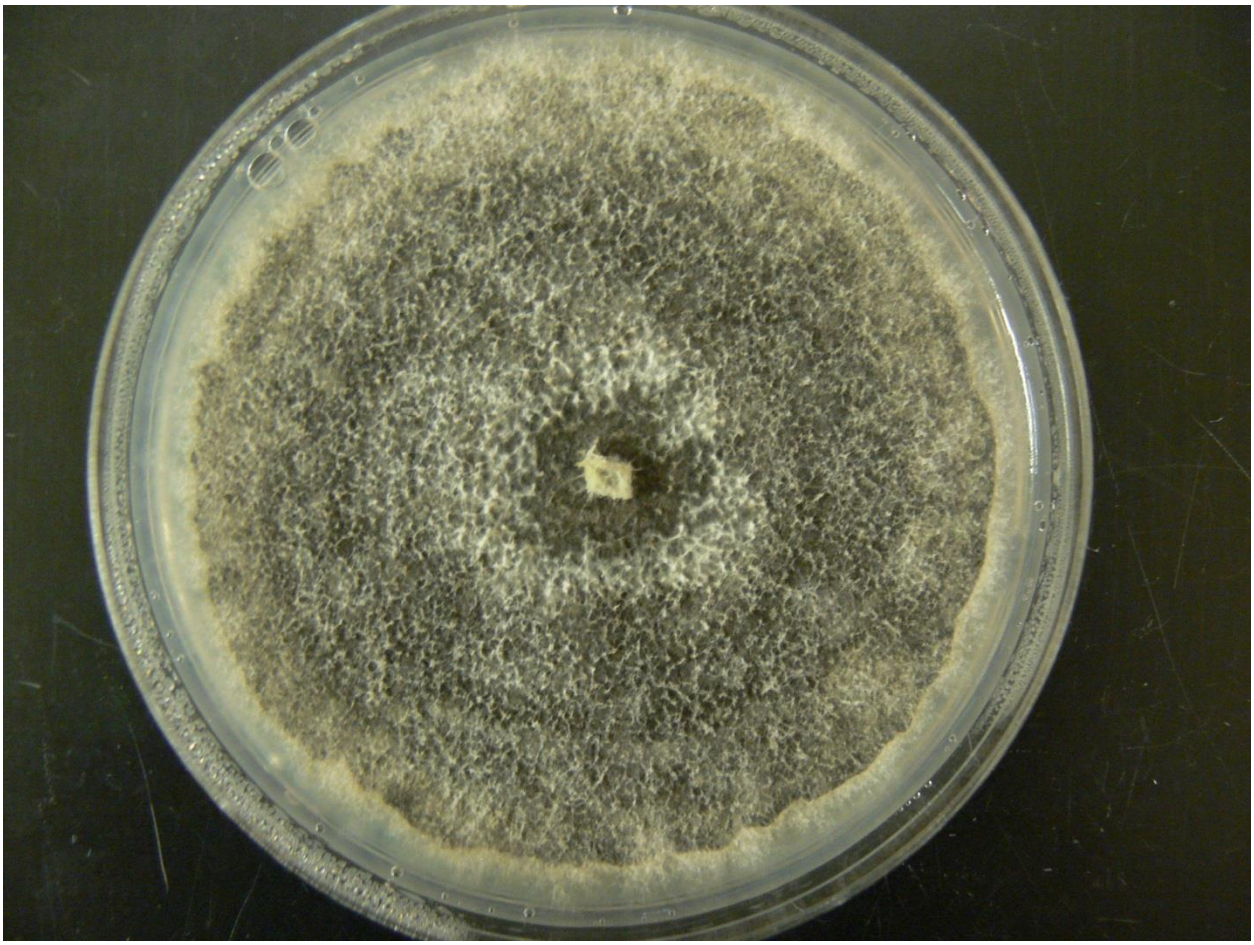

Figure 2.11. Front view of IL12-Ds-3 on APDA.

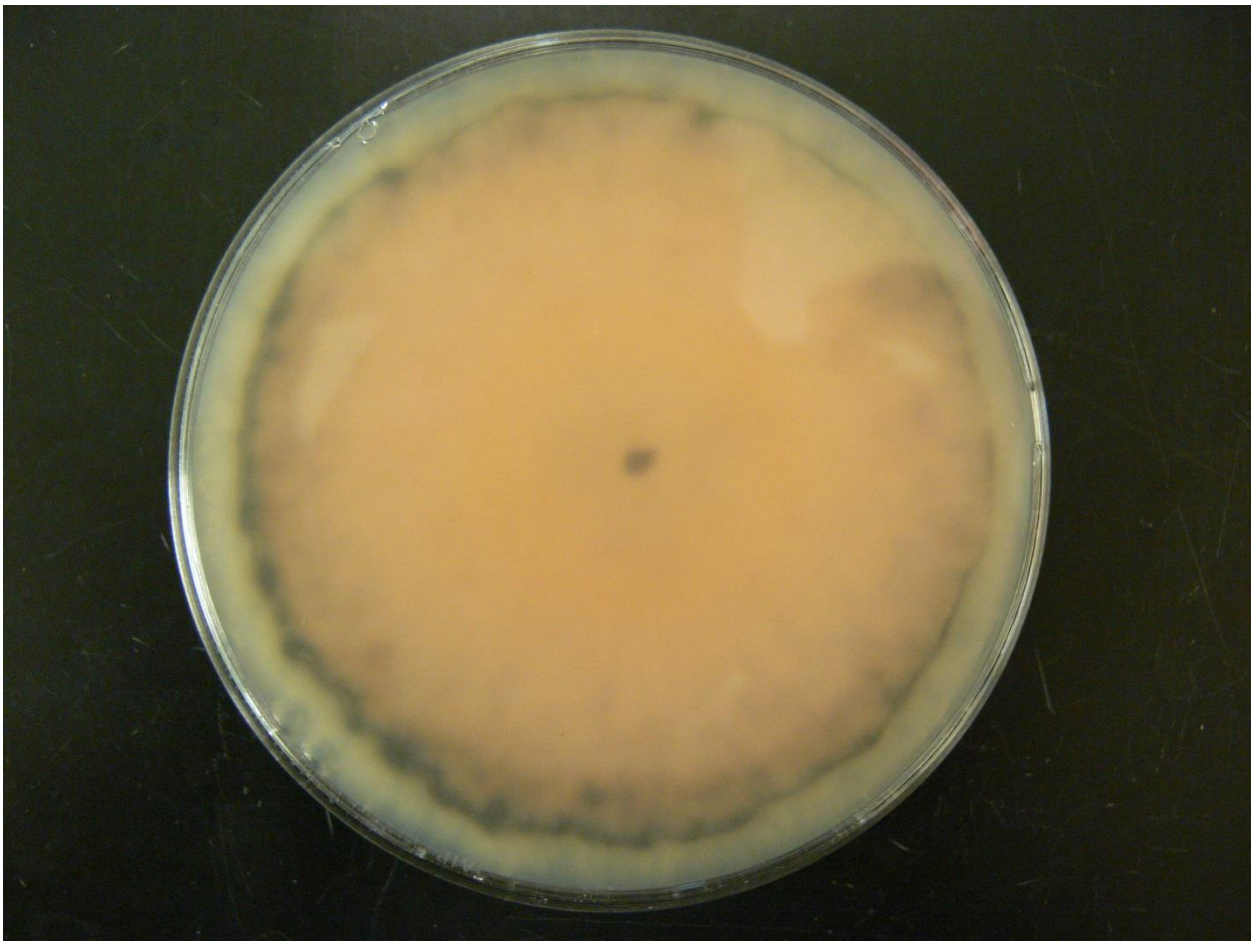

Figure 2.12. Reverse of IL12-Ds-3 on APDA.

The sequenced ITS region of the subcultured and unsubcultured IL12-Ds-1 isolates and of the IL12-Ds-2 isolate revealed that all three isolates had 100% sequence identity. BLAST alignment revealed that 26 sequences from GenBank had 100% identity with the sequence, 24 of which were reported to be *P. longicolla* sequences or its synonyms, and three of which were reported to be ambiguous *Phomopsis*/*Diaporthe* spp. Phylogenetic analysis of IL12-Ds-1 and fungi listed in the Zhang et al. (1998) paper revealed that IL12-Ds-1 was in the grouping containing *P. longicolla* and *D. phaseolorum* var. *sojae* (Figure 2.13). *D. phaseolorum* var. *caulivora* and *D.*

phaseolorum var. *meridionalis* were still monophyletic taxa, and two *D. phaseolorum* var. *sojae* isolates formed an additional clade not seen in the original analysis by Zhang et al. (1998).

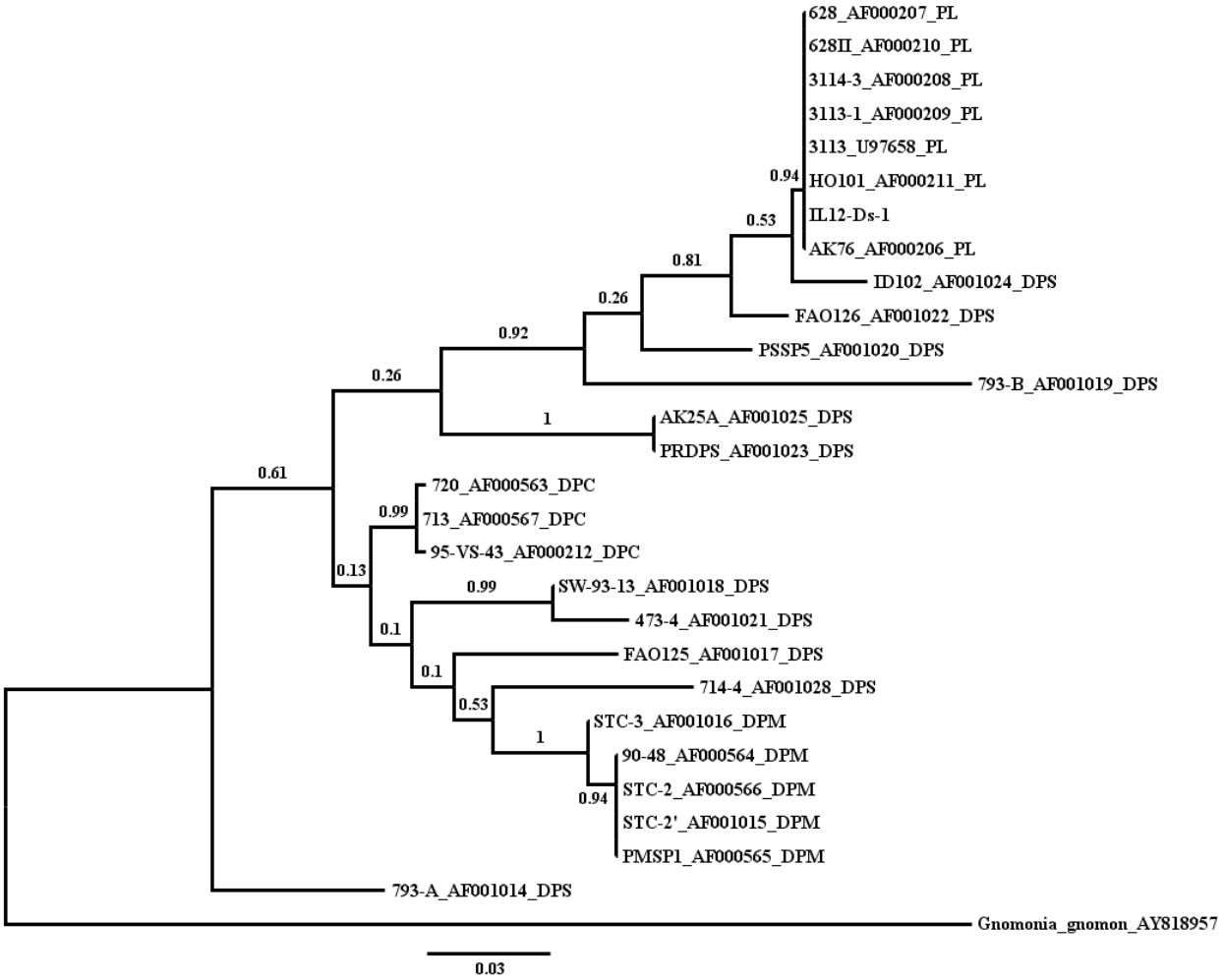

Figure 2.13. A phylogenetic tree visualized using FigTree 1.4 (Rambaut 2012) with the tips indicating isolate codes from Zhang et al. (1998), isolate GenBank accession numbers, and the isolate species designation according to Zhang et al. (1998) from left to right and separated by underscores. Isolate species designations are PL, DPS, DPC, and DPM for *P. longicolla*, *D. phaseolorum* var. *sojae*, *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis*, respectively. A sequence from *Gnomonia gnomon* was used as the outgroup. Bootstrap proportions are given beside the branches. The scale bar units are nucleotide substitutions per site.

Discussion

Initially, the cultural characteristics of IL12-Ds-1 seemed to suggest that the isolate was *D. phaseolorum* var. *sojae* rather than *P. longicolla* as pycnidial beak length plays a prominent role in the Hobbs et al. (1985) taxonomy (the epithet "longicolla" is derived from a combination of the Latin words *longus* [long] and *collum* [neck]). However, by the seventh subculture, except for the presence of variable proportions of beta-conidia and the simple and rarely branched conidiophores, the isolate matched the *P. longicolla* descriptors (Hobbs et al. 1985). Mean alpha-conidial width and L/W ratio from pycnidia developed on APDA did not provide useful information for determining whether the isolate was more likely to be *P. longicolla* or *D. phaseolorum* var. *sojae* as those values did not match up with values expected for either species, although the mean alpha-conidial length was close to the one described for *P. longicolla*. Conidial dimensions were not determined before the seventh subculture of IL12-Ds-1, so it is not known if any of the dimensions changed as a result of the subculturing. Although BLAST alignment of the sequenced IL12-Ds-1 ITS region suggested that the isolate was *P. longicolla,* phylogenetic analysis grouped the isolate with both *P. longicolla* and *D. phaseolorum* var. *sojae* isolates. In terms of pathogenicity, IL12-Ds-1 has been shown to cause PSD and pod and stem blight but not stem canker (Chapter 3). The features of this isolate verify the morphological flexibility of the *Diaporthe*/*Phomopsis* complex and its environmental component. Additionally, our findings support the concept that *P. longicolla* and *D. phaseolorum* var. *sojae* are actually the same species as both of these species cannot be distinguished morphologically, pathogenically, or phylogenetically.

Due to the inadequacies of the Hobbs et al. (1985) taxonomic descriptors and to avoid future ambiguity between members of the DPC, a phylogenetic approach is proposed to classify fungi

that appear to be part of the DPC. Initially, the ITS region of the fungus should be sequenced and a phylogenetic tree created using the isolates in the Zhang et al. (1998) paper. If the fungus groups with either *D. phaseolorum* var. *caulivora* or *D. phaseolorum* var. *meridionalis*, the fungus should be regarded as the taxon with which it groups as both of these fungi form monophyletic groups. However, the names *Diaporthe caulivora* (Athow & Caldwell) J.M. Santos, Vrand. & A.J.L. Phillips and *Diaporthe aspalathi* E. Jansen, Castl. & Crous should be used instead of *D. phaseolorum* var. *caulivora* or *D. phaseolorum* var. *meridionalis*. The use of the varietal rank signifies the existence of distinct morphological characteristics between groups of isolates of a species, and as previously discussed, such distinct characteristics do not exist in the DPC (Morgan-Jones 1989). If the fungus does not group with either of those two species, it should be regarded as *D. sojae*, which is likewise of the species rank.

Changes in cultural morphology of *D. sojae* with subculturing have been previously reported with some isolates. Kmetz (1975) found that the proportion of beta-conidia in *D. sojae* pycnidia increased with successive subcultures, although this was not observed in all isolates. It should be pointed out that subculturing of IL12-Ds-1 was not temporally successive, and the seven subcultures were carried out over a period of about one year. Moreover, Kmetz et al. (1975) did not report how many cultures were observed by for a particular subculture, although the number had to be at least two (the number of replications in the experiment), and it is possible that the increased proportion of beta-conidia observed with the successive subcultures was due to the inherent intra-cultural variability of the fungus as observed in IL12-Ds-1. Therefore, it is likely that the changes in cultural morphology of IL12-Ds-1 are due to an accumulation of mutations or epigenetic changes that are inherited due to the artificial environment under which the fungus is grown in rather than subculturing. This can be seen from the single-alpha-conidial isolate from

the non-subcultured isolate of IL12-Ds-1 that still had morphology that was not seen in cultures before the fourth generation of subculturing, viz., effuse stromata. However, it is also possible that these morphologic changes might be due to the culturing of selected heterokaryotic hyphae of IL12-Ds-1. These changes appeared to be irreversible as neither reisolating the fungus from soybean leaves on water agar nor reisolating the fungus from soybean seed from plants inoculated with IL12-Ds-1 (Chapter 3) reversed the changes.

The inability of beta-conidia of IL12-Ds-1 to develop inside pycnidia when incubated in a plastic container on a laboratory shelf seems to suggest a more complex mechanism at play than nutrient depletion as has heretofore been reported as mycelial growth was more vigorous in cultures incubated in a plastic container on a laboratory shelf. It would therefore be expected that nutrient depletion, and subsequent formation of beta-conidia, would have occurred in the pycnidia of such cultures (Jensen 1983; Morgan-Jones 1985). Light intervals, temperature, and humidity can possibly play a role in activating the mechanism of beta-conidial production in *D. sojae*. The failed attempts at eliciting beta-conidial production artificially using cold temperature incubation methods that were successfully used by Vidić et al. (2013) show that inter-isolate variability in beta-conidial production mechanisms exist. Our failed attempts to germinate beta-conidia of IL12-Ds-1 follow other unsuccessful attempts at beta-conidial germination and give more weight to the idea that beta-conidia of the DPC are either functional or relictual spermatia rather than spores (Hildebrand 1954; Jensen 1983; Lehman 1923; Morgan-Jones 1985; Wehmeyer 1933).

The intermediate spore type formed by pycnidia of IL12-Ds-1 on soybean leaves fits the description given to gamma conidia, which have been described as “hyaline, multiguttulate, fusiform to subcylindrical with an acute or rounded apex, while the base is sometimes truncate” (Udayanga et al. 2011). Conidia intermediate between alpha and beta-conidia have previously

been reported to be formed by *D. phaseolorum* var. *sojae* and *D. phaseolorum* var. *caulivora* (Hildebrand 1954; Luttrell 1947; Wehmeyer 1933), although Hildebrand (1954) found them to be biguttulate while the ones found in the present investigations were multiguttulate. It is not yet known whether the gamma conidia of the DPC are able to germinate. As gamma conidia have been previously found to be formed by *D. phaseolorum* var. *sojae* and *D. phaseolorum* var. *caulivora*, they cannot be used to differentiate fungus species in the DPC, and although gamma conidial formation by *D. phaseolorum* var. *merdionalis* has yet to be reported, the rarity of such conidial formation in all members of the DPC is likely the reason for this.

The cultural morphology of IL12-Ds-2 is novel to members of the DPC. Interestingly, a recently identified species, *Diaporthe beilharziae* R.G. Shivas, J. Edwards & Y.P. Tan, seems to have a similar morphology on PDA, viz., abundant pycnidia, no perithecia, and inconspicuous white mycelia (Tan et al. 2013). Alpha-conidia dimensions of IL12-Ds-2 were not considerably different from those found from IL12-Ds-1 alpha-conidia. Due to the unusual cultural morphology of this isolate, it is possible that this morphology was overlooked or possibly misidentified as *Alternaria* spp. by researchers during soybean seed assays. On the other hand, it is possible that this alternate morphology is the result of a mutation or epigenetic change produced by culturing IL12-Ds-1. The IL12-Ds-2 morphology was also observed on soybean seed from plants that had been inoculated with IL12-Ds-1 (Chapter 3). While this was not a common observation, it shows the propensity for morphological change by IL12-Ds-1 to not be confined to laboratory conditions. However, IL12-Ds-2 has yet to be observed undergoing morphological change as neither single-alpha-conidial isolations nor temporally successive subculturing altered its morphology. It has yet to be determined whether IL12-Ds-2 is more

virulent or aggressive in causing PSD or pod and stem blight, and extensive field tests with this fungus should not be conducted before such information is known.

The cultural morphology of IL12-Ds-3 is also atypical compared to members of the DPC, although the occurrence of cultures lacking fruiting bodies has previously been reported (Mengistu et al. 2007). In the Mengistu et al. (2007) study, the isolates lacking the ability to form pycnidia were hypovirulent. Therefore, it is possible that IL12-Ds-3 has a mutation that causes hypovirulence. Our method for isolating IL12-Ds-3 is a novel method for conducting rapid single-spore isolations for members of the DPC lacking the ability to produce pycnidia or perithecia. The method can also be used to obtain large amounts of inoculum from such fungi for the purpose of inoculating plants.

Chapter 3: Screening Soybean Genotypes for Resistance to Phomopsis Seed Decay Using Various Assays

Introduction

Phomopsis seed decay (PSD), a fungal disease that causes disfiguration, molding, and pre-emergence damping-off of soybean (*Glycine max* (L.) Merr.) seed, can be caused by any member of the *Diaporthe*/*Phomopsis* complex (DPC), which consists of *Diaporthe sojae* Lehman, which is synonymous with *Phomopsis longicolla* and *D. phaseolorum* var. *sojae*, *D. caulivora* (Athow & Caldwell) J.M. Santos, Vrand. & A.J.L. Phillips, which is synonymous with *D. phaseolorum* var. *caulivora*, and *Diaporthe aspalathi* E. Jansen, Castl. & Crous, which is synonymous with *D. phaseolorum* var. *meridionalis*, with *D. sojae* being the most common causal pathogen (Gomes et al. 2013; Sinclair 1999). The classification of the members of the DPC was recently treated (Chapter 2). Screening of soybean plant introductions (PIs) and cultivars for PSD resistance has commonly been performed in the field (Brown et al. 1987; Li et al. 2011; Zimmerman and Minor 1993), although Roy and Abney (1988) did perform a small (six pots per trial) experiment in the greenhouse using a misting chamber to promote PSD. More recently, screening of soybean genotypes has been performed in the greenhouse, and a comparison of PSD incidence in the greenhouse and PSD incidence in the field suggests that greenhouse assays are better able to detect an accession's true resistance level due to the greater ability to control the environmental conditions (Sun et al. 2012a).

Screening for PSD resistance has usually consisted of spraying plants with *D. sojae* conidia during the seed development stages or allowing natural infection to occur, and subsequently plating mature seed on potato dextrose agar (PDA) to observe fungal growth and determine disease incidence. Both of these methods require the harvesting of seed at harvest maturity with

or without a delay. Given the length of time needed to grow plants to harvest maturity, it would be more economical to screen plants when they are immature and, moreover, with a non-destructive assay. Zhang and Xue (2014) recently developed a non-destructive detached leaf assay for PSD resistance, but the utility of this assay remains to be demonstrated as the reported experiments had no resistant or susceptible controls to compare the tested cultivars against. Inoculating immature stems of soybeans has been a common method of screening for resistance to stem canker, which is primarily caused by caused *D. caulivora* and *D. aspalathi* (Crall 1952; Keeling 1982). Such a stem assay, however, has not been attempted to screen for PSD resistance, although *D. sojae* has been shown to cause lesions when stem-inoculated (Chao and Glawe 1985; Li et al. 2010). Screening soybean accessions without the need for planting would be another way to shorten the time it takes to identify PSD-resistant accessions. A mature seed inoculation assay, which has been used to determine the aggressiveness of DPC members in causing seed decay, has yet to be used in screening for PSD resistance (Kmetz et al. 1974; Kmetz 1975).

The objectives for this series of experiments described here were (i) to develop and evaluate a greenhouse assay using the traditional inoculation and screening procedures but with a modification that allows a large number of diverse soybean genotypes to be efficiently screened for PSD resistance at a high density, (ii) to test whether assays involving inoculation of an immature stem or mature seed can be used to screen for PSD resistance, and (iii) to assess the utility of a detached leaf assay as a method of screening for PSD resistance.

Materials and Methods

An overview of all the experiments conducted is given in Table 3.1.

Table 3.1. An overview of the experiments conducted.

Assay	Harvest Season	Inoculation Method[1]	Inoculum Type	Average Temp (°C)[2]	Average Relative Humidity (%)[2]	Environmental Changes[3]
Standard 1	Summer	Spraying (2)	Spore suspension	28	70	None
Standard 1 – RIL	Summer	Spraying (2)	Spore suspension	28	70	None
Standard 2	Winter	Spraying (3)	Spore suspension	23	40	Misting + Watering + Covering
Standard 2 – RIL	Winter	Spraying (3)	Spore suspension	23	40	Misting + Watering + Covering
Standard 3 – RIL	Spring	Spraying (5)	Spore suspension	27	50	Misting + Covering
Seed 1	N/A	Toothpick tip (1)	Mycelia	22	70	Moist paper towel
Stem 1	Winter	Toothpick tip (1)	Mycelia	23	40	None
Stem 2	Spring	Toothpick tip (1)	Mycelia	27	50	Misting
Leaf 1	Spring	Pipetting (1)	Mycelia/spore suspension	22	70	Moist paper towel
Leaf 2	Spring	Pipetting (1)	Mycelia	22	70	Moist paper towel

[1] The number within the parenthesis signifies the number of times the inoculation method was performed.
[2] The average temperatures and relative humidities are those post-inoculation.
[3] Misting was either done manually (Standard 2, Standard 2 – RIL) or using an automatic misting system (Standard 3 – RIL, Stem 2). Watering means that plants were watered twice instead of once a day, and covering refers to the use of a plastic sheet to cover plants overnight to maintain high humidity and high temperature and to decrease the photoperiod.

Screening diverse genotypes using the standard seed plating assay (Standard)

On 21 February 2013, 21 soybean plant introductions (PIs) and cultivars were planted in six sand flats (Table 3.2). Some of the PIs and cultivars selected served as resistant and susceptible checks while all others were selected due to their parentage in available mapping populations. Six thick, rigid, unpartitioned, 53 × 38 × 11 cm plastic greenhouse flats with drainage holes in the bottom (Kadon Corp., Dayton, OH) were filled to the top with steam-sanitized torpedo sand. Before the flats were filled, their bottoms were lined with paper towels to prevent the sand from leaking through the drainage holes. Seven 2.5 cm deep trenches were made across each flat with a specifically designed tool. Each of the seven rows was divided into two by placing a plastic marker stake at the halfway point. Six seeds of each PI or cultivar were deposited in the trench and covered with sand. The experimental design was an incomplete split plot in an incomplete unbalanced block design with maturity group being the split-plot factor. Maturity group was either early (MG II to VII) or late (MG VII to IX). There were four replications of the PIs and cultivars. Plants were watered once a day. Daytime, nighttime, and average relative humidity post-inoculation in the greenhouse room were 82%, 57%, and 70%, respectively. Daytime, nighttime, and average temperatures post-inoculation in the greenhouse room were 31°C, 24°C, and 28°C, respectively.

Table 3.2. Accessions used in the Standard experiments.

Accession	Maturity Group	Origin	PSD Resistance/Susceptibility Reference
'5601T'	V	TN, USA	Li et al. (2011)
'Amsoy 71'	II	IN, USA	Roy and Abney (1988)
'Benning'	VII	GA, USA	
'Dwight'	II	IL, USA	
LD00-3309	IV	IL, USA	
LD02-4485	II	IL, USA	
LG00-3372	III	IL, USA	
PI 203398	VIII	Brazil (Japanese ancestry)	
PI 416834	IX	Japan	
PI 417132	VII	Japan	
PI 417208	VIII	Japan	
PI 417479	IV	Japan	Brown et al. (1987)
PI 506947	VIII	Japan	
PI 567046A	VIII	Indonesia	
PI 567058D	IX	Indonesia	
PI 567085B	VIII	Indonesia	
PI 567104B	IX	Indonesia	
PI 567129	IX	Indonesia	
PI 567139B	IX	Indonesia	
PI 567238	IX	China	
PI 605823	IX	Vietnam	
PI 80837	IV	Japan	Yelen and Crittenden (1967)
PI 91113	III	China	Zimmerman and Minor (1993)

Plants were inoculated with IL12-Ds-1 on 24 April 2013 with 5×10^5 colony forming units (CFU) mL^{-1} and a second time on 6 May 2013 with 1×10^5 alpha-conidia mL^{-1}. An inoculum concentration of 1×10^5 alpha-conidia mL^{-1} was chosen based on a previous experiment in which a *D. sojae* inoculum concentration of 9×10^4 conidia mL^{-1} gave a significant increase in the recovery of *D. sojae* from trifoliate leaf disks and stem pieces compared to lower inoculum concentrations, and higher inoculum concentrations failed to increase the recovery of *D. sojae* (Jackson 2000). Inoculations occurred when most plants were at the R2 through R6

developmental stages, which correspond to full flowering and fully developed green seeds, respectively (Fehr et al. 1971). The first inoculum suspension was prepared by removing the growing IL12-Ds-1 cultures that had not yet formed pycnidia from the Petri dishes, blending them in sterile double-distilled water using a Waring blender, and then filtering the suspension through four layers of cheesecloth. Hemocytometer readings of the filtrate were then recorded. The second inoculum suspension was prepared by flooding IL12-Ds-1 culture plates containing pycnidia with sterile double-distilled water, waiting 10 min, and then rubbing the pycnidia with a sterile glass rod. The suspension from the plates was collected in one storage bottle and then filtered through four layers of cheesecloth into another. Hemocytometer readings of the filtrate were then recorded. For both inoculations, the filtered suspensions were poured into a 4-L garden sprayer (Chapin International, Inc., Batavia, NY) and diluted with double-distilled water to get the final concentrations. 125 µl L^{-1} of enzyme grade Tween 20 (Thermo Fisher Scientific Inc., Waltham, MA) were added to the final solution. Leaves, stems, and pods were sprayed until runoff at 14:30 on 24 April 2013 and at 17:00 on 6 May 2013. After the first inoculation, 15 cm transparent plastic domes were put on the top of the flats during the evening and were left overnight until the next morning to maintain a high relative humidity around the plants to promote infection; however, this was discontinued after three days due to sun damage to the foliage on especially sunny mornings. Plants were harvested at the R8 stage of development. The increasing natural photoperiod during maturation caused some accessions to revert to vegetative growth even though there were already pods at harvest maturity on the plants. Because flowering occurred over a period of several weeks, some pods reached harvest maturity while a second flush of immature pods were still developing. The older pods were harvested as they matured to reduce the risks of shattering and seed germination inside pods that were still attached to plants.

The vegetative stem tips of plants that reverted to vegetative growth were removed, and only yellow pods were allowed to finish maturing. With the intent of maximizing PSD on the seeds, plants were cut approximately 4 cm above the sand level with pruning shears and were placed in paper bags with other plants from the same replication. The harvested material was kept in a storage room at approximately 24°C until all of the plants had been harvested, and then the paper bags containing the harvested plants were returned to the more humid environment of the greenhouse room for five days to promote fungal growth on the seed. Afterward, the pods were hand-threshed, and the seed were surface disinfected as described above prior to being plated on acidified potato dextrose agar (APDA) at a density of five to ten seeds per plate, depending on seed size. On average, 35 seeds were plated per replication (excluding replications with fewer than five seed). APDA was prepared from Difco™ PDA (Becton, Dickinson and Company, Franklin Lakes, NJ) amended with 800 µL of 85% lactic acid (Thermo Fisher Scientific Inc., Waltham, MA) per liter to lower the pH of the agar to 4.5. All Petri dishes were incubated in an Adaptis A350 growth chamber with a CMP6010 control system (Conviron, Winnipeg, Canada). The temperature was maintained at 22 ± 1°C, the relative humidity was maintained at 70 ± 5%, the average light intensity was maintained at 75 µmol m^{-2} s^{-1}, and the light period was set to 12 hours light and 12 hours dark. Light intensity was measured by putting a MQ-200 Quantum Meter (Apogee Instruments, Inc., Logan, UT) on top of a Petri dish in three different locations on each of the three shelves and taking the average of the readings. Fungi were identified based on cultural morphology, viz., mycelia color and texture, and vigor of mycelial growth, after seven days of incubation. Fungi with morphology unlike that of *D. sojae* were grouped into the miscellaneous fungi category. Bacterial infection was determined based on observed symptoms and signs.

On 17 September 2013, the same 21 soybean PIs and cultivars were planted in six sand flats, with the exception of PI 203398 and PI 567085D. These were replaced with PI 417479 and PI 605823 to add another PSD-resistant accession and to include an accession that was a parent of a recombinant inbred line (RIL) population that was simultaneously being screened the Standard 2 experiment while still keeping the number of flats constant. Additionally, three sand flats containing plants from each of the entries served as uninoculated controls. The experimental design for the experiment was a completely randomized design with four replications for the experimental group and two replications for the control group. The planting conditions and the watering regimen before inoculation with IL12-Ds-1 were the same as above. Inoculation with IL12-Ds-1 occurred on 13 and 24 November 2013 at 17:00 and 16:00, respectively, and on 14 December 2013 at 16:00 with a concentration of 1×10^5 alpha-conidia mL^{-1} for all three inoculations. Inoculations occurred when most plants were at the R2 through R6 developmental stages. At the same time, the uninoculated controls were mock-inoculated by being misted with water. After the first inoculation, a thin, black, opaque plastic sheet was used to cover both the experimental and control groups daily at 16:00 until the end of harvest in an attempt to increase the relative humidity around the plants (Figure 3.1). The sheet was taken off at 09:00 daily. Additionally, the water regimen was changed to include misting, another watering, and another misting two hours, four hours, and six hours, respectively, after the initial watering at 09:00. Plants were harvested seven days after reaching the R8 (full maturity) developmental stage. For this experiment, only pods were harvested rather than whole plants, with the pods being hand-threshed. Additionally for this experiment, seed were surface disinfected by placing them in 0.5% sodium hypochlorite for 4 min and then rinsing them once in sterile double-distilled water before they were plated on APDA at a density of five to ten seeds per plate depending on seed

size. In addition to being simpler, this surface disinfection protocol has been shown to provide a more accurate representation of the fungi that exist on seed than the one used in the Standard 1 experiment, which consisted of using ethanol and sodium hypochlorite (Grybauskas et al. 1979; Yorinori and Sinclair 1983). On average, 21 seeds were plated per replication (excluding replications with fewer than five seed). Fungal and bacterial identification methodology was the same as above. Daytime, nighttime, and average relative humidity post-inoculation in the greenhouse room were 44%, 36%, and 40%, respectively. Daytime, nighttime, and average temperature post-inoculation in the greenhouse room were 26°C, 20°C, and 23°C, respectively.

Figure 3.1. A thin, black, opaque plastic sheet used to cover plants in the Standard 2, Standard 2 – RIL, and Standard 3 – RIL experiments.

Data for all experiments were analyzed using SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC). PROC MIXED and PROC GLM were used in the Standard 1 and Standard 2 experiments, respectively, for ANOVA. The degrees of freedom were corrected with the

Kenward-Roger approximation for the Standard 1 experiment. Multiple comparisons of the soybean genotypes were performed with the Tukey-Kramer adjustment under the LSMEANS statement. PROC CORR was used to obtain the Pearson's correlation coefficients between PSD incidence, days to maturity, miscellaneous fungal infection, and bacterial infection. Any replicate with fewer than five seeds was excluded from the analysis. This was done in order to avoid inaccurate PSD incidence values stemming from low seed counts. A p-value of 0.05 was used to determine statistical significance.

Screening RILs using the standard seed plating assay (Standard – RIL)

On 14 March 2013, 118 lines from the $F_{4:5}$ generation of a population derived from LD02-4485 (MG II) × PI 417208 (MG VII) along with four replications of both parents were planted in nine sand flats as described in the Standard 1 experiment that was conducted at the same time period as this experiment. On 15 May 2013 and 30 May 2013, all plants were inoculated with 8.5×10^4 alpha-conidia mL^{-1} and 1×10^5 alpha-conidia mL^{-1}, respectively, of the IL12-Ds-1 isolate as explained in the Standard 1 experiment. On both days, most plants were at the R2 through R6 developmental stages. Plants were harvested and seed were assayed as described for the Standard 1 experiment. On average, 31 seeds were plated per replication (no replications had fewer than five seed). The experimental design was a completely randomized design with no replications, with the exception of the parents.

On 17 September 2013, 87 lines from the $F_{4:5}$ generation of a population derived from 5601T (V) × PI 605823 (IX), along with five replications of both parents, were planted in seven sand flats as previously explained. Inoculation, relative humidity management (covering, watering, and misting plants), and harvest protocols were the same as in the Standard 2 experiment that was

conducted during the same time period as this screening experiment. Inoculations occurred when most plants were at the R2 through R6 developmental stages. The experimental design was a completely randomized design with no replications, with the exception of the parents.

On 23 December 2013, 87 lines from the $F_{4:5}$ generation of a population derived from 5601T (V) × PI 605823 (IX), along with 11 replications of both parents, were planted in 14 sand flats as previous explained in a greenhouse room equipped with automatic misters. The experimental design was a completely randomized design with two replications. Plants were covered from 09:00 to 16:00 daily from the beginning of the experiment using the same material described previously. Plants were watered daily until the first inoculation. Plants were thinned to three plants in each half-row two weeks after planting, and a 45 cm bamboo stake was put next to each plant for trellising to create a more uniform microenvironment. Plants were inoculated between 16:00 and 17:00 with 1×10^5 alpha- and beta-conidia mL^{-1} on 18 February, 28 February, and 4 March 2014 and with 1×10^5 alpha-conidia mL^{-1} on 25 March and 28 March 2014. Inoculations occurred when most plants were at the R2 through R6 developmental stages. Starting after the first inoculation, plants were uncovered one hour later, i.e., at 10:00, to keep relative humidity higher for a longer period of time. The morning after the first inoculation after the plants were uncovered, automatic misters were turned on to mist for eight seconds every eight minutes. The only time misters were turned off after this time was from two hours before each of the other four inoculations until the following morning. This was done to reduce the possibility of washing away the inoculum. Manual watering was halted after the first inoculation because misting alone provided enough water for the plants. Plant height was measured before any harvesting occurred as the length from the soil surface to the node containing the upper-most pod. Pods were harvested singly from plants at a weekly interval once they were at harvest maturity because the

pods did not mature simultaneously on some plants, and waiting for younger pods to mature would have put undue disease pressure on the already mature pods and would increase the possibility of germination of seeds within the mature pod. Green pods that developed from a second flush of flowers were not harvested. Harvest date was recorded as the date that the last pod was harvested from a plant. Harvested pods were examined for evidence of pycnidia, one of the symptoms of pod and stem blight. Seed were plated on APDA following the revised surface disinfection protocol described above, and fungi and bacteria were identified as described for the Standard 1 experiment. On average, 17 seeds were plated per replication (excluding replications with fewer than five seed). Daytime, nighttime, and average relative humidity post-inoculation in the greenhouse room were 56%, 44%, and 50%, respectively. Daytime, nighttime, and average temperature post-inoculation in the greenhouse room were 29°C, 25°C, and 27°C, respectively.

PROC GLM in SAS was for ANOVA, and in the Standard 3 – RIL experiment, to determine if any of the lines had any form of resistance to PSD, a one-tailed Dunnett's test was performed with 5601T as the resistant control under the LSMEANS statement. PROC CORR was used to obtain the Pearson's correlation coefficients between PSD incidence, days to maturity, miscellaneous fungal infection, bacterial infection, and percentage of pods with pycnidia. Any replicate with fewer than five seeds was excluded from the analysis of the Standard 3 – RIL experiment. A p-value of 0.05 was used to determine statistical significance.

Screening diverse genotypes using a mature seed inoculation assay (Seed)

Mature seed of two PSD-resistant PIs, PI 417479 and PI 360841, and two PSD-susceptible PIs, PI 91113 and PI 360835, were surface disinfected by placing them in 0.5% sodium hypochlorite for 4 min and then rinsing them once in sterile double-distilled water. They were then soaked in

sterile double-distilled water for 60 min, placed in Petri dishes lined with moist paper towels at a density of five seeds per plate, inoculated by sticking < 1 cm of a quill toothpick end covered with IL12-Ds-1 into a puncture made using a 1 mm-wide dissecting needle (Thermo Fisher Scientific Inc., Waltham, MA; Catalog #08-965-A), and incubating them for eight days in the growth chamber. Paper towels were rehydrated four days after initial incubation. Toothpick ends were covered by IL12-Ds-1 by placing them pointed side up on the edge of a growing culture of IL12-Ds-1 on APDA for three days. Mock-inoculation control toothpick ends were placed on culture-free APDA plates for the same time period. After incubation, seed were determined to either be non-germinated or germinated. Non-germinated seed were either completely covered with mycelia or had emerged radicles less than 1.5 times the length of the seed while germinated seed had emerged radicles at least 1.5 times the length of the seed. The above method is a modification of Crall's (1952) toothpick inoculation method sensu Kmetz et al. (1974) and Kmetz (1975). There were three replications of the experimental group and one replication of the control group. The first two experimental replications and the control replication had 25 seeds each while the third experimental replication had 21, 10, 25, and 15 seeds for PI 417479, PI 360841, PI 91113, and PI 360835, respectively.

PROC GLM in SAS was used for ANOVA, and the LSMEANS statement was used to obtain multiple comparisons with the Tukey-Kramer adjustment to see if any of the varieties in the experimental group had significantly different seed germination failure rates. A p-value of 0.05 was used to determine statistical significance.

Screening diverse genotypes using an immature stem assay (Stem)

On 7 March 2014, four replications, three experimental and one uninoculated control, of PI 417479 and PI 91113, a genotype resistant and susceptible to PSD, respectively, were planted in plastic flats in a completely randomized design as described above, with the exception that LC1 Sunshine Mix (Sun Gro Horticulture Canada Ltd., Agawam, MA) was substituted for torpedo sand, and five seeds were planted per replication. After three weeks, a < 1 cm quill toothpick end covered with IL12-Ds-1 was inserted into the stem 3 mm below the first trifoliate node after a puncture was made using a dissecting needle. The toothpick ends were prepared as described above but were incubated for two days instead of three. Mock-inoculation control toothpick ends were prepared as described above. After seven days, plant stems were harvested and lesions were photographed using an Olympus DP70 digital camera (Olympus America Inc., Center Valley, PA) attached to an Olympus SZX12 stereo microscope, and their lengths were measured using ImageJ 1.47 (Rasband 1997-2014).

On 28 May 2014, four experimental replications and two control replications of 5601T, LD00-2817P, PI 209908, PI 360841, PI 417479, PI 605823, and PI 91113 were planted in plastic flats in a completely randomized design as described for the Stem 1 experiment. 5601T, PI 209908, PI 360841, and PI 417479 were the PSD-resistant genotypes, and LD00-2817P, PI 605823, and PI 91113 were the PSD-susceptible genotypes. After two weeks, plants were thinned to three per replication, and a < 1 cm quill toothpick end covered with IL12-Ds-1 was inserted into the stem 3 mm below the first trifoliate node after a puncture was made using a dissecting needle. The toothpick ends were prepared as described above with an incubation time of three days. Mock-inoculation control toothpick ends were prepared as described above. Misters were turned on the morning following inoculation for eight seconds every 16 minutes until the end of the

experiment. After seven days, lesion size and plant height, which was measured as the length of the stem from the soil line to the upper-most node, were measured.

PROC GLM in SAS was used for ANOVA, with either lesion size or plant height as the dependent variable. The SLICE statement was used to obtain least square mean differences in lesion size between the varieties when they were inoculated, and multiple comparisons with the Tukey-Kramer adjustment were calculated to see if any of the varieties had significantly different lesion sizes for the Stem 1 and Stem 2 experiments, respectively. PROC CORR was used to obtain the Pearson's correlation coefficient between height and lesion size. A p-value of 0.05 was used to determine statistical significance.

Screening diverse genotypes using a detached leaf assay (Leaf)

At the end of the Stem 2 experiment, three fully expanded trifoliate leaves from the highest node with fully expanded leaves were harvested along with their supporting petiole from one three-week-old plant in each replicate and were placed in a Ziploc® bag with a moist sterile paper towel inside for transport to the laboratory. Each leaf was placed on a moist sterile paper towel in a Petri dish, and a 0.5 mm hole was made in the center at the main leaf vein using a dissecting needle. Each leaf was then inoculated by pipetting the inoculum on the hole. Inoculum consisted either of 50 µl of 1×10^5 CFU mL^{-1} of IL12-Ds-1, 50 µl of 1×10^5 alpha-conidia mL^{-1} of IL12-Ds-1, or 50 µl of sterile double-distilled water, which served as the control. The first treatment was prepared following the protocol laid out by Zhang and Xue (2014). The mycelia-containing portions of five five-day-old cultures of IL12-Ds-1 were cut out and placed in a Waring blender with 150 ml of sterile double-distilled water. The blender was turned on for one minute, and CFU concentration was determined by serial dilution of the suspension and subsequent plating of

the dilutions on APDA. The CFU concentration was determined to be 1×10^5 CFU mL^{-1}, a concentration more than 1000-fold less than that obtained by Zhang and Xue (2014). The suspension was stored at 4°C until use. Each one of the three trifoliate leaves of each of the four experimental and two control replicates of each of the seven soybean varieties received one of the three treatments after which the Petri dishes were closed and placed in the growth chamber in a completely randomized design. After six days, necrotic lesion area and percent necrotic lesion area out of total leaf area was calculated using ImageJ. In order to determine if the trifoliate leaves from either the plants stem-inoculated with IL12-Ds-1 or the mock-inoculated plant were infected by IL12-Ds-1 prior to our inoculation via the petioles, each petiole from which trifoliate leaves were harvested from was cut up into three segments, and the segments were rinsed in sterile double-distilled water, plated on APDA, and observed for fungal growth.

The detached leaf assay experiment was repeated on four-week-old detached leaves from the same group of plants. The protocol was the same as the Leaf 1 experiment, except that all three trifoliate leaves from one replication were treated with 100 µl of 4×10^5 CFU mL^{-1}, which was the inoculum concentration derived from blending two 15-day-old plates of IL12-Ds-1 for one minute in 50 mL of sterile double-distilled water. The concentration was determined by serial dilution of the suspension and subsequent plating of the dilutions on APDA. The experiment was a completely randomized design with the three trifoliate leaves harvested considered to be subsamples within each replication. After six days, no necrotic lesions formed, but a reddish discoloration appeared on the veins of the trifoliate leaves with the main vein showing the most prominent discoloration. The length of the discoloration along the main vein from the abaxial side of the leaf, as well as the length of the leaf, was measured for the trifoliate leaves tested.

PROC GLM was used for ANOVA in both experiments, and the LSMEANS statement was used to conduct multiple comparisons with the Tukey-Kramer adjustment. Both lesion area and percent lesion area and discoloration length and percent discoloration length were used as response variables for the Leaf 1 and Leaf 2 experiments, respectively. A p-value of 0.05 was used to determine statistical significance.

Results

Screening diverse genotypes using the standard seed plating assay (Standard)

In the Standard 1 experiment, the results of the ANOVA showed that the effect of blocks on PSD incidence was not significant, but the effect of variety, which was nested in maturity group, was significant. Multiple comparisons using the Tukey-Kramer adjustment did not show any significant differences among the mean PSD incidences of the various varieties (Table 3.3). No significant correlations between PSD incidence, days to maturity, miscellaneous fungal infection, or bacterial infection were found, with the exception of a significant low positive correlation between miscellaneous fungal infection and bacterial infection (Table 3.4).

Table 3.3. All accessions tested in the Standard 1 and Standard 2 experiments with their corresponding least square means for Phomopsis seed decay (PSD) incidence. Only replications with five or more seed were included in the analysis. Least square means for PSD incidence followed by the same letter within a column are not significantly different using the Tukey-Kramer adjustment in multiple comparisons. N/A signifies that an accession was not tested or did not have at least one replication.

	PSD Incidence (%)	
Accession	Standard 1	Standard 2
PI 417208	23 a	0 ab
LD02-4485	20 a	0 ab
PI 567104B	16 a	2 ab
Benning	15 a	5 ab
PI 203398	13 a	N/A
PI 91113	13 a	0 b
PI 567046A	12 a	0 ab
PI 567238	12 a	6 ab
LD00-3309	11 a	2 ab
PI 417132	6 a	0 ab
Amsoy 71	5 a	0 ab
PI 567139B	5 a	6 ab
PI 416834	4 a	N/A
PI 567085B	4 a	0 ab
PI 567129	4 a	3 ab
5601T	3 a	N/A
PI 567058D	3 a	N/A
LG00-3372	1 a	0 ab
PI 80837	1 a	N/A
Dwight	0 a	1 ab
PI 506947	-1 a	0 ab
PI 417479	N/A	13.5 a
PI 605823	N/A	0 ab

Table 3.4. Pearson's correlation coefficients for the Standard 1 experiment between Phomopsis seed decay (PSD) incidence, days to maturity, miscellaneous fungal infection, and bacterial infection. An asterisk signifies that a correlation is statistically significant ($p \leq 0.05$).

	PSD Incidence (%)	Days to Maturity	Miscellaneous Fungal Infection (%)	Bacterial Infection (%)
PSD Incidence (%)	1	0.06	0.12	0.05
Days to Maturity		1	-0.02	0.16
Miscellaneous Fungal Infection (%)			1	0.25*
Bacterial Infection (%)				1

In the Standard 2 experiment, overwatering led to a loss of many replicates, forcing the exclusion of some genotypes from the analysis. ANOVA showed that the effect of variety on PSD incidence was significant. Multiple comparisons using the Tukey-Kramer adjustment of the remaining varieties showed that PI 417479 had significantly greater PSD incidence than PI 91113 (Table 3.3). This was unexpected as PI 417479 is a known resistant genotype and PI 91113 is a known susceptible genotype. All other varieties had PSD incidences that were not significantly different from one another. There were no significant correlations between PSD incidence, days to maturity, miscellaneous fungal infection, or bacterial infection, with the exception of a significant moderate positive correlation between miscellaneous fungal infection and days to maturity (Table 3.5). No PSD was found on seed harvested from any of the mock-inoculation controls.

Table 3.5. Pearson's correlation coefficients for the Standard 2 experiment between Phomopsis seed decay (PSD) incidence, days to maturity, miscellaneous fungal infection, and bacterial infection. An asterisk signifies that a correlation is statistically significant ($p \leq 0.05$).

	PSD Incidence (%)	Days to Maturity	Miscellaneous Fungal Infection (%)	Bacterial Infection (%)
PSD Incidence (%)	1	-0.15	-0.19	0.004
Days to Maturity		1	0.54*	-0.002
Miscellaneous Fungal Infection (%)			1	-0.09
Bacterial Infection (%)				1

Screening RILs using the standard seed plating assay (Standard – RIL)

In the Standard 1 – RIL experiment, LD02-4485 had no PSD, and only one replication of PI 417208 had a PSD incidence similar to that found in the Standard 1 experiment. As LD02-4485 and PI 417208 were the two most PSD-susceptible lines found in that experiment, and disease incidence was extremely low on seeds from 46 RILs that were evaluated (65% had no PSD and 93% had a PSD incidence of 10% or less), it was not possible to obtain useful data for genetic mapping; hence, seed from the remaining 63 RILs were not plated and evaluated. The low PSD incidence indicated that conditions for disease development were not adequate.

In the Standard 2 – RIL experiment, most plants did not survive due to overwatering, and those that did survive only produced one or two pods. From this experiment also, it was not be possible to obtain useful data for genetic mapping, so no seed were plated to assess PSD incidence.

In the Standard 3 – RIL experiment, the ANOVA results showed that the effect of genotype (RILs and their parents) was significant, and PSD incidence was higher than 86% in 90% of the genotypes. Only one RIL was found to have significantly less PSD than 5601T using a one-tailed Dunnett's test, and PI 605823 was not found to have a significantly different PSD incidence than

5601T using the same test. PSD incidence was significantly negatively correlated with miscellaneous fungal infection and bacterial infection (Table 3.6). PSD incidence was not significantly correlated with days to maturity, height, or the incidence of pycnidia on pods. The lack of a significant correlation between PSD incidence and days to maturity indicated that inoculation of plants with different maturity periods at the same time was not a problem. There were also low but significant positive correlations between days to maturity and height, the incidence of pycnidia on pods and bacterial infection, and miscellaneous fungal infection and bacterial infection. Additionally, there was a low but significant negative correlation between days to maturity and bacterial infection.

Table 3.6. Pearson's correlation coefficients for the Standard 3 – RIL experiment between Phomopsis seed decay (PSD) incidence, days to maturity, miscellaneous fungal infection, and bacterial infection. An asterisk signifies that a correlation is statistically significant ($p \leq 0.05$).

	PSD Incidence (%)	Days to Maturity	Height	Pycnidia on Pods	Miscellaneous Fungal Infection (%)	Bacterial Infection (%)
PSD Incidence (%)	1	0.12	0.14	0.09	-0.75*	-0.57*
Days to Maturity		1	0.18*	-0.07	-0.05	-0.24*
Height			1	0.08	-0.14	0.01
Pycnidia on Pods				1	0.01	0.16*
Miscellaneous Fungal Infection (%)					1	0.27*
Bacterial Infection (%)						1

Screening diverse genotypes using a mature seed inoculation assay (Seed)

The Seed 1 experiment showed a significant difference in the percentage of ungerminated seed between the inoculated seed and the mock-inoculated control seed of both the resistant and susceptible cultivars, but there were no significant differences between the experimental groups of the four soybean varieties. Three germinated seed of the second experimental replication of PI

91113 and one germinated seed of the first experimental replication of PI 360835, which constituted all of the germinated seed in the experimental groups, escaped infection due to dislodgment of the toothpick tip during handling. Excluding these escapes, none of the seed in the experimental groups germinated. All ungerminated seed of the experimental groups were completely covered with white mycelia characteristic of IL12-Ds-1. None of the germinated or ungerminated seed of the control groups were covered with mycelia.

Screening diverse genotypes using an immature stem assay (Stem)

In the Stem 1 and Stem 2 experiments, stem inoculation significantly increased lesion size, which was larger for the second inoculation experiment, and height was not significantly correlated with lesion size. In the Stem 1 experiment, PI 91113 did not have a significantly different lesion size from PI 417479, and in the Stem 2 experiment, the only significant difference was between PI 91113 and PI 209908, with the lesion size of the latter being smaller (Table 3.7). It should be noted that PI 209908 experienced delayed germination compared with the rest of the genotypes tested.

Table 3.7. Least square means of the lesion size, lesion area and percent lesion area out of total leaf area, and discoloration length and percent discoloration length out of total leaf length for the Stem 1 and Stem 2, Leaf 1, and Leaf 2 experiments, respectively. Least square means followed by the same letter within a column are not significantly different using the Tukey-Kramer adjustment in multiple comparisons.

	Stem 1	Stem 2	Leaf 1		Leaf 2	
Accession	Lesion Size (mm)		Lesion Area (mm^2)	Lesion Area (%)	Discoloration Length (mm)	Discoloration Length (%)
PI 91113	2.1 a	6.0 b	88 a	4 a	40 ab	47 ab
PI 417479	2.0 a	5.0 ab	70 a	4 a	46 b	55 ab
5601T	N/A	4.6 ab	72 a	3 a	30 a	38 a
LD00-2817P	N/A	4.5 ab	135 a	5 a	36 ab	48 ab
PI 605823	N/A	4.3 ab	89 a	3 a	35 ab	43 ab
PI 360841	N/A	4.3 ab	26 a	1 a	46 b	58 b
PI 209908	N/A	3.2 a	102 a	7 a	38 ab	55 ab

Screening diverse genotypes using a detached leaf assay (Leaf)

In the Leaf 1 experiment, only the mycelia-inoculated leaves formed lesions, and neither the effect of variety nor stem inoculation was significant whether the response variable used was lesion area or percent lesion area (Table 3.7). In the Leaf 2 experiment, the effect of variety and the interaction between variety and stem inoculation were significant whether the response variable was discoloration length or percent discoloration length. No veinal discoloration appeared on the trifoliate leaves inoculated with a spore suspension or sterile double-distilled water in the Leaf 1 experiment; therefore, the veinal discoloration was thought to be the result of the mycelial inoculation. Only 5601T exhibited a significantly different response to stem inoculation, and stem inoculation in this cultivar increased both discoloration length and percent discoloration length. Discoloration length of 5601T was significantly less than that of PI 360841 or PI 417479, and percent discoloration length of 5601T was significantly less than that of PI 360841 (Table 3.7). No fungal growth resembling IL12-Ds-1 was found from plated petiole segments in either the Leaf 1 or Leaf 2 experiments. This means that the trifoliate leaves assayed were not infected with mycelia of IL12-Ds-1 from the stem inoculation.

Discussion

The main difficulty in conducting greenhouse assays using the standard agar plate assay is in the optimization of environmental conditions necessary to obtain levels of PSD adequate to discern susceptible genotypes from resistant genotypes without overwhelming the resistant plants. Only inoculating plants and letting them mature in a moderately humid greenhouse room was not enough, as the uninformative results from the Standard 1 and Standard 1 – RIL experiments show. As relative humidity has been shown to be more important in increasing PSD incidence in

growth chamber tests (Spilker et al. 1981) and as the Standard 3 – RIL experiment showed that extremely high levels of PSD can be achieved without manipulating temperature apart from the increased temperature due to covering the plants with a plastic sheet, relative humidity should be the main factor manipulated in greenhouse assays to achieve a high level of PSD. The results of the Standard 3 – RIL experiment also showed that IL12-Ds-1 was effective in causing PSD as long as environmental conditions were suitable for the promotion of the disease. A risk associated with attempting to increase relative humidity is the possibility of overwatering when temperatures are low in a greenhouse room, a condition exacerbated when maturation occurs during winter, as exemplified by the Standard 2 and Standard 2 – RIL experiments. Keeping plants in a room with an average temperature of over 26°C would reduce the risk of this happening. Balducchi and McGee (1987) recommended maintaining environmental conditions suitable for optimum growth of *D. sojae* (100% relative humidity and 25°C) for three consecutive days to achieve high levels of PSD; therefore, it is recommended that conditions similar to these are put in place after each inoculation. It should be noted, however, that Balducchi and McGee (1987) used detached pods to determine that three consecutive days of 100% relative humidity and 25°C caused greater than 90% of seed to have PSD. Attached pods might be more resistant as the plants can elicit a molecular immune response to inhibit pod infection or seed infection by *D. sojae*. Multiple inoculations are needed as diverse genotypes or RILs do not mature simultaneously. Environmental conditions suitable for PSD development should not be maintained indefinitely as resistance to PSD can be overcome as was seen for 5601T, a cultivar with resistance to PSD (Li et al. 2011), in the Standard 3 – RIL experiment. It is also possible that the very high levels of PSD were due to pathogen aggressiveness or virulence as suggested by the high degree of aggressiveness seen in the mature seed inoculation

assay and the result of the Standard 2 experiment where PI 417479, a PSD-resistant genotype had the highest incidence of PSD, but the overwhelming amount of escapes, as seen in the lack of PSD on susceptible genotypes, makes this inference unlikely without additional experiments being undertaken. Additionally, race-specific resistance to PSD has yet to be reported.

Although the effect of covering the plants with plastic sheets was not tested in the experiments conducted, if one wants to keep plants at 100% relative humidity for three days, covering the plants at night, which is when relative humidity and temperature usually drop, would be ideal. In addition to increasing relative humidity and temperature, covering the plants also reduces the photoperiod, further helping to reduce the time between planting to harvesting. In addition, care must be taken to inspect pods during harvest as seed germination in the pod can result in lost seed, and this is especially important in the greenhouse as plants grown in sand had a tendency to experience staggered intra-plant pod maturation as seen from the third RIL screening experiment. Furthermore, harvest should not be delayed when relative humidity has been artificially increased, e.g., by misting, as it might allow *D. sojae* to overcome a resistant plant's defense mechanisms.

Another factor that might influence PSD incidence is the inoculum concentration used and the number of applications of inoculum in an experiment. In addition to the inoculum experiments conducted by Jackson (2000), which did not look into PSD incidence, Seo et al. (2009) found that DPC isolate inoculum concentrations of 1.55×10^7 conidia mL^{-1} produced "clearer" symptoms than inoculum concentrations of 1.55×10^5 conidia mL^{-1} and 1.55×10^6 conidia mL^{-1}. Due to the vagueness of the abstract and the unavailability of a full corresponding research article, it was not possible to ascertain which member(s) of the DPC was used in these experiments or what symptoms were actually seen and made "clearer" by the higher inoculum

concentrations. Furthermore, if an inoculum suspension contains both alpha- and beta-conidia, only alpha-conidia should be used in calculating the inoculum concentration as beta-conidia have been shown not to germinate and are likely either functional or relictual spermatia rather than spores (Chapter 2; Hildebrand 1954; Jensen 1983; Lehman 1923; Morgan-Jones 1985; Wehmeyer 1933).

The lack of a significant correlation between PSD and pycnidia on pods in the Standard 3 – RIL experiment, when taking into account previous research done on the relationship between PSD and pod and stem blight (Hepperly and Sinclair 1980a; Kmetz et al. 1978; Prasartsee et al. 1975), provides further evidence for the unreliability of trying to estimate a plant's PSD incidence on the basis of pod and stem blight symptoms. The significant negative correlation in the Standard 3 – RIL experiment between PSD and miscellaneous fungal infection and bacterial infection is likely the result of antagonism between the fungi and bacteria that infected the seed and *D. sojae.* Antagonism between various fungi and bacteria and *D. sojae* has previously been reported by several research groups (Begum et al. 2008; Cubeta et al. 1985; Hepperly et al. 1983; Hepperly and Sinclair 1981; Hilty 1993; Manandhar et al. 1987a; Manandhar et al. 1987b; McGee et al. 1980; Pathan et al. 1989; Roy and Abney 1977; Yeh and Sinclair 1980). Although pods closer to the base of a soybean are more likely to be infected by *D. sojae* (Kmetz et al. 1978), the Standard 3 – RIL experiment showed no correlation between plant height and PSD. It is possible that the overcoming of resistance distorted the relationship between pod height and PSD. The lack of a significant correlation in the Standard 3 – RIL experiment between PSD and days to maturity is likely due to the relatively constant environmental conditions inherent in a greenhouse experiment. The lack of such a correlation in soybeans grown in the tropics corroborates this theory (Paschal and Ellis 1978); however, a significant negative correlation has also been found

between days to maturity and PSD in RILs segregating for PSD resistance in the greenhouse (Sun et al. 2013).

Unlike resistance to foliar and root diseases that can be screened using seedlings, PSD resistance screening typically requires that plants reach harvest maturity before screening via seed plating can begin. The alternative assays used to screen for PSD resistance without having to wait for harvest maturity do not seem promising, with the possible exception of the immature stem inoculation assay. The Seed 1 experiment demonstrated that mature seed of resistant genotypes cannot inhibit colonization by *D. sojae* when inoculated. The Leaf 1 and Leaf 2 experiments did not identify any susceptible genotypes having significantly greater lesion areas or discolored lengths than the resistant genotypes. While Zhang and Xue (2014) found 20 resistant genotypes out of 74 tested, they did not correct for multiple comparisons, which might be one reason why they reported significant differences among the cultivars tested. Additionally, without resistant and susceptible checks, it was not known whether the significant differences were due to general pathogen resistance or the same resistance mechanism found in PSD-resistant genotypes. The results of the present studies suggest that the former is likely the case. The Stem 2 experiment found a resistant genotype, PI 209908, with a significantly smaller lesion size than a susceptible genotype, PI 91113; however, the rest of the resistant and susceptible accessions had lesion sizes that were not significantly different. This suggests that either the immature stem assay tests a non-specific immune response or that a lack of ideal environmental conditions, viz., high humidity and high temperature, prevented a better separation of means. It is also possible that delayed germination of PI 209908 caused this genotype to be more resistant to colonization of *D. sojae*. It should be noted that the immature stem inoculation assay cannot detect resistance

mechanisms that are based on physiology rather than biochemistry, and this might limit the utility of such an assay if pod physiology is found to play a role in PSD resistance.

Chapter 4: Mycoparasitism and Biocontrol of *Diaporthe sojae* by *Acremonium strictum*

Introduction

Diaporthe sojae Lehman, which is synonymous with *Phomopsis longicolla* and *D. phaseolorum* var. *sojae*, is the primary cause of Phomopsis seed decay (PSD), a soybean (*Glycine max* (L.) Merr.) seedborne fungal disease that reduces seed quality (Gomes et al. 2013; Kulik and Sinclair 1999). The classification of the members of the *Diaporthe*/*Phomopsis* complex was recently treated (Chapter 2). In order to reduce the impact of this disease, a variety of cultural and chemical management strategies exist, such as delayed planting and fungicide use, but a biological control for this disease has yet to be developed. Although studies on the antagonism of *D. sojae* by other fungi and bacteria exist, many of those microorganisms are pathogens of soybean themselves, which make them unlikely candidates for biocontrol of PSD (Begum et al. 2008; Cubeta et al. 1985; Hepperly et al. 1983; Hepperly and Sinclair 1981; Hilty 1993; Manandhar et al. 1987a; Manandhar et al. 1987b; McGee et al. 1980; Pathan et al. 1989; Roy and Abney 1977; Yeh and Sinclair 1980). In a study by Manandhar et al. (1987b), saprobic fungi did not have an effect on germination of soybean seed inoculated by *D. sojae* in a greenhouse trial; however, there was an increase in germination in field trials, but as the stand count of seed inoculated with *D. sojae* and *Colletotrichum truncatum* was not separately compared against the control, an inference on the biocontrol potential of the saprobic fungi on *D. sojae* cannot be made. Saprobic fungi, including an *Acremonium* sp., and non-pathogenic bacteria have, however, been found to inhibit *D. sojae* in vitro (Begum et al. 2008; Manandhar et al. 1987b).

Acremonium strictum W. Gams, a fungus that can act as a plant pathogen (Bandyopadhyay et al. 1980; Chase and Munnecke 1980; Kang and Singh 1976; Natural et al. 1982; Racedo et al. 2013; Simay 1988), an endophyte (McGee et al. 1991), a saprobe (Domsch et al. 2007), and a

mycoparasite (Choi et al. 2008; Rivera-Varas et al. 2007), has yet to be isolated from soybean under natural conditions but has been recovered from artificially inoculated soybeans (Chase and Munnecke 1980). No symptoms developed on the artificially inoculated soybeans. Moreover, *A. strictum* along with verlamelin, a compound extracted from an *A. strictum* isolate, have been found to inhibit growth of various plant pathogenic fungi in vitro and in vivo (Choi et al. 2008; Choi et al. 2009; Kim et al. 2002; McGee et al. 1991).

The objectives for present series of experiments were (i) to investigate the mycoparasitism of *D. sojae* by *A. strictum* and (ii) to investigate the biocontrol potential of *A. strictium* against *D. sojae* in vitro, in vivo, and ex vivo.

Materials and Methods

Isolation of *D. sojae* and *A. strictum*

A single-spore isolate of *D. sojae*, named Li53, isolated from velvetleaf in Champaign, IL in 2000 was sent to us from Stoneville, MS by Dr. Shuxian Li of the USDA-ARS. Long-beaked pycnidia and effuse stroma were observed in the culture, but most of the pycnidia were empty and those that were not produced spores that were not typical of *D. sojae*. When isolation of such spores was attempted, orange colonies developed rather than colonies exhibiting typical *D. sojae* mycelial growth. One of the orange isolates was used for later experimentation and was eventually named As-1. Subculturing the Li53 isolate produced cultures devoid of pycnidia. In order to produce a single-spore isolate from a culture that lacked pycnidia, a stromatal plug from Li53 was placed on water agar with soybean leaf pieces that had been autoclaved for 30 minutes at 121°C. Once pycnidia formed on the leaves, those leaves were placed in a test tube with sterile double-distilled water, the test tube was vortexed, and the conidial suspension was spread on an

APDA plate using a bacterial loop. A 1 mL NORM-JECT® Luer lock syringe (Henke-Sass, Wolf GmbH, Tuttlingen, Germany) with an 18Gx1 (1.2 mm x 25 mm) PrecisionGlide™ needle (Becton, Dickinson and Company, Franklin Lakes, NJ) was then used to pick up a single-alpha-conidium with the syringe tip and place it on a clean APDA plate with circles made with a marker to signify where the syringe tip should be moved to displace the conidia for later confirmation of isolation success. The syringe tip was flame-sterilized after each single-alpha-conidial isolation attempt.

Identification of As-1 using morphology and ITS1 and ITS4 primers

Genomic DNA of As-1 grown on acidified potato dextrose agar (APDA) was extracted using a FastDNA™ SPIN Kit (MP Biomedicals, Santa Ana, CA). APDA was prepared from Difco™ PDA (Becton, Dickinson and Company, Franklin Lakes, NJ) amended with 800 µL of 85% lactic acid (Thermo Fisher Scientific Inc., Waltham, MA) per liter to lower the pH of the agar to 4.5. Amplification with the polymerase chain reaction (PCR) was performed on a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) using 1.5 mM 10X PCR buffer with 15mM $MgCl_2$, 0.1 mM dNTPs, 0.1 µM of both ITS1 and ITS4 primers (White et al. 1990), 1 unit Taq (Bioline Reagents Ltd, London, UK), and 200 ng μl^{-1} DNA with a total volume of 25 µl per reaction. The PCR settings were 94°C for 5 min, 35 cycles of 94°C for 45 sec, 55°C for 1 min, and 72°C for 1 min. The PCR product was purified using a QIAquick® PCR Purification Kit (Qiagen, Venlo, The Netherlands) and was then sent to the W.M. Keck Center for Comparative and Functional Genomics' High-Throughput Sequencing and Genotyping Unit for fragment analysis on a 3730xl DNA Analyzer (Life Technologies Corporation, Carlsbad, CA). Sequences were created from raw nucleotide data using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, MI), which included trimming the outer portions of the sequences containing low quality

data and manually ascertaining the identity of nucleotides found ambiguous by the program. The sequenced ITS region was then aligned against the sequences in the National Center for Biotechnology Information's (NCBI) GenBank® using NCBI's Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990; Benson et al. 2008). After obtaining the results of the BLAST alignment, conidiophore morphology, spore dimensions, and presence of chlamydospores were examined under the microscope. Spores were photographed using an Olympus DP70 digital camera (Olympus America Inc., Center Valley, PA) attached to an Olympus BX51 microscope using a phase-contrast lens, and their dimensions were measured using ImageJ 1.47 (Rasband 1997-2014).

Sectoring induction assay

In order to see if sectoring of *D. sojae* can be induced by As-1 so that a culture can be produced that would lack the ability to produce pycnidia, a mycelial plug of a *D. sojae* isolate (IL12-Ds-1) previously isolated from soybean seed was placed in the center of an APDA plate and spores of As-1 were spread in a circle to surround the IL12-Ds-1 plug. After mycelia of the two fungi overgrew each other, the mycelia at the interface were cut out of the APDA plate and placed either on APDA or on water agar with autoclaved soybean leaf pieces. Single-spore isolations were conducted on the latter culture as described above. Single-spore isolate and non-single-spore isolate cultures were examined for pycnidial development.

In vitro assays

In order to assess the width of the zone of inhibition between IL12-Ds-1 and As-1, a protocol similar to the one by Royse and Ries (1978) was followed. A 4 mm mycelial plug of As-1 cut with a cork borer from the edge of a growing As-1 culture on APDA was placed mycelial-side

down 1 cm from the periphery of an APDA plate. After five days of incubation in a growth chamber, a 4 mm mycelial plug from the edge of a growing five-day old culture of IL12-Ds-1 was placed 5 cm from the mycelial plug of As-1 mycelial-side down. All Petri dishes were incubated in an Adaptis A350 growth chamber with a CMP6010 control system (Conviron, Winnipeg, Canada). The temperature was maintained at 22 ± 1°C, the relative humidity was maintained at 70 ± 5%, the average light intensity was maintained at 75 µmol m^{-2} s^{-1}, and the light period was set to consecutive 12 hours light and 12 hours dark intervals. Light intensity was measured by putting a MQ-200 Quantum Meter (Apogee Instruments, Inc., Logan, UT) on top of a Petri dish in three different locations on each of the three shelves and taking the average of the readings. There were four replications of both incubation intervals. After eight, nine, and ten days, the width of the zone of inhibition between the two fungi was determined according to Royse and Ries (1978).

In order to determine what effects the chemical compounds released by As-1 have on IL12-Ds-1 mycelial growth, a mycelial plug of As-1 was placed in an Erlenmeyer flask containing 175 mL of Difco™ Potato Dextrose Broth (PD broth) (Becton, Dickinson and Company, Franklin Lakes, NJ), and the flask top was subsequently covered with plastic wrap and incubated in the growth chamber. After 25 days, the culture filtrate of As-1 was obtained by filtering the broth using a 500 mL vacuum-driven 0.22 µm nylon membrane filter (Argos Technologies, Inc., Elgin, IL). Water agar plates were made that contained either 25%, 15%, or 5% of the culture filtrate, and water agar plates without any of the culture filtrate served as a control. A 4 mm mycelial plug of IL12-Ds-1 was placed in the center of each of the plates in the dilution series. There were three replications for each plate in a completely randomized design. Mycelial growth was measured as

the average of the longest length of mycelial growth observed from the reverse and its perpendicular. Mycelial growth was measured for six days.

In order to determine if the zone of inhibition observed can be induced by the chemical compounds released by As-1, a 1 cm filter paper disc was dipped in either 100% or 50% of the culture filtrate and placed 1 cm from the periphery of an APDA plate. Sterile double-distilled water was used as the control. A 4 mm mycelial plug from the edge of a growing five-day old culture of IL12-Ds-1 was placed 5 cm from the soaked filter paper disc. After ten to 12 days, the width of the zone of inhibition between IL12-Ds-1 and the soaked filter paper disc was determined.

In order to determine how IL12-Ds-1 alpha-conidia are affected by the chemical compounds released by As-1, 2×10^6 alpha-conidia mL^{-1} of IL12-Ds-1 suspended in PD broth were diluted 50% with either 100% of the culture filtrate or 50% of the culture filtrate (diluted with sterile double-distilled water), and the suspension was incubated in the growth chamber under the same environmental conditions that were described previously. Sterile double-distilled water acted as the control diluent. After 24 hours, the percentage of alpha-conidial germination was determined using a hemocytometer. An alpha-conidium was considered to have germinated when the germ tube was at least half its length. There were three replications for each culture filtrate concentration and the control in a completely randomized design.

In vivo and ex vivo assays

Antagonism of IL12-Ds-1 by As-1 on soybean seed was investigated by inoculating seeds of 'Williams 82' with either IL12-Ds-1, As-1, or a combination of IL12-Ds-1 and As-1 by soaking seed for one hour in a suspension of either 1×10^6 alpha-conidia of IL12-Ds-1 mL^{-1}, 1×10^6

conidia of As-1 mL^{-1}, or 1×10^6 alpha-conidia of IL12-Ds-1 and 1×10^6 conidia of As-1 mL^{-1}, respectively, and letting the seed air-dry overnight before planting them in soil or placing them in Petri dishes as described below. Sterile double-distilled water was used as the control treatment. The seed to be planted were planted in two thick, rigid, unpartitioned, 53 × 38 × 11 cm plastic greenhouse flats with drainage holes in the bottom (Kadon Corp., Dayton, OH). These flats were filled to the top with LC1 Sunshine Mix (Sun Gro Horticulture Canada Ltd., Agawam, MA), after their bottoms were lined with paper towels to prevent the potting media from leaking through the drainage holes. Seven 2.5 cm deep trenches were made across each flat with a specifically designed tool. Each of the seven rows was divided into two by placing a plastic marker stake at the halfway point. Five seeds of Williams 82 were deposited in the trench and covered with the potting mix. The experimental design was a completely randomized design with six replications of each of the treatments including the control. The flats were misted for eight seconds every 16 minutes. After seven days, seed rot was recorded for each of the five seeds. Samples of ten seeds were placed in Petri dishes lined with moist sterilized paper towels. The experimental design was a completely randomized design with five replications of each of the treatments including the control. After seven days, germination frequencies were recorded. A seed was considered germinated if the length of its radicle was at least 1.5 times the length of the seed.

Antagonism of IL12-Ds-1 by As-1 on soybean leaves was investigated by inoculating autoclaved, 1 cm^2 pieces of Williams 82 leaves with either IL12-Ds-1, As-1, or a combination of IL12-Ds-1 and As-1 by soaking the leaves for one hour in a suspension of either 1×10^6 alpha-conidia of IL12-Ds-1 mL^{-1}, 1×10^6 conidia of As-1 mL^{-1}, or 1×10^6 alpha-conidia of IL12-Ds-1 and 1×10^6 conidia of As-1 mL^{-1}, respectively. They were then air-dried overnight, and placed

on water agar plates at five leaf pieces per dish. The plates were then placed in a growth chamber in a completely randomized design with three replications per treatment. Sterile double-distilled water was used as the control treatment. After four days, leaves exhibiting any pycnidial development were counted. In addition, leaves with 50% or greater pycnidial coverage on the surface were counted.

SAS Enterprise Guide 6.1 (SAS Institute Inc., Cary, NC) was used to conduct all data analysis. PROC GLM was used to analyze all experiments except the mycelial growth experiment on culture filtrate dilution plates where PROC MIXED was used. For all experiments except the mycelial growth experiment on culture filtrate dilution plates, PROC GLM was used for ANOVA and multiple comparisons of least square means were performed with the Tukey-Kramer adjustment under the LSMEANS statement. For the mycelial growth experiment, PROC MIXED was used with mycelial growth data treated as repeated measures, and multiple comparisons with the Tukey-Kramer adjustment were carried out to see if any of the culture filtrate dilution plates differed in mycelial growth for each day. The covariance structure used was AR(1), and the degrees of freedom were corrected with the Kenward-Roger approximation. A p-value of 0.05 was used to determine statistical significance in all experiments.

Results

The BLAST alignment of the sequenced ITS region of As-1 determined that ITS sequences reported to be from *Nectria mauritiicola* (Henn.) Seifert & Samuels, *Acremonium kiliense* Grütz, and *Acremonium strictum* had 100% identity with As-1. Morphologically, the isolate had simple conidiophores, mean conidial dimensions of 4.5 × 2.1 µm with a range of 2.6-6.1 × 1.3-3.4 µm based on 100 conidia, and no chlamydospores. As this morphology matches the description of *A.*

strictum (Domsch et al. 2007), with the exception that As-1 has a slightly larger conidial width, the fungus was determined to be *A. strictum*. *Acremonium kiliense* forms chlamydospores and *Nectria mauritiicola* forms perithecia and synnemata; therefore, both of these fungi were eliminated as possibilities in the identification of As-1 (Domsch et al. 2007; Seifert 1985).

All single-conidial isolates of Li53 produced effuse stroma and no pycnidia. Single-conidial isolate and non-single-conidial isolate cultures derived from the culture of mycelia from the interface of IL12-Ds-1 and As-1 failed to sector sensu Li53, and in cultures of IL12-Ds-1 surrounded by As-1, pycnidia with conidia typical of *D. sojae* developed even if they formed close to the interface between the two fungi.

The average zone of inhibition between IL12-Ds-1 and As-1 after eight days was 4.5 mm, but it decreased to 2 mm and 1 mm after nine and ten days (Table 4.1). At the tenth day, three of the four replicates had overlapping mycelial growth from the two fungi. No zone of inhibition developed around any of the filter paper disc treatments and IL12-Ds-1 grew unabated. For the culture filtrate dilution plate experiment, no significant inhibition of growth of IL12-Ds-1 was seen for any plates until the fifth day compared to the control (Table 4.2). Only the 25% culture filtrate plates on the fifth and sixth days significantly inhibited IL12-Ds-1 mycelial growth; however, significant differences were found amongst the culture filtrate dilution plates starting from the fourth day. No significant differences in percent alpha-conidial germination were observed between any of the incubated culture filtrate or control spore suspensions (Table 4.3). In the seed inoculation assays, inoculation with As-1 did not provide a significant protective effect against IL12-Ds-1 in the greenhouse or in the growth chamber when compared to the positive control, which was the IL12-Ds-1 inoculation treatment (Table 4.4). As-1 did not significantly decrease germination in either the greenhouse or growth chamber experiment when

compared to the negative control, which was the sterile double-distilled water treatment. In the leaf inoculation assay, As-1 did not significantly inhibit development of pycnidia on soybean leaves, and the IL12-Ds-1- inoculated leaves and the combined IL12-Ds-1 and As-1-inoculated leaves had the same mean values for presence of pycnidia on leaves and the presence of leaves with 50% or greater pycnidial coverage on the surface area, with the values being 73% and 87%, respectively.

Table 4.1. Zones of inhibition between IL12-Ds-1 and As-1 in each of the four replications and their averages for the eighth, ninth, and tenth days. A value of 0 indicates that the mycelial growth of the two fungi overlapped.

	Zone of Inhibition (mm)		
Replication	Day 8	Day 9	Day 10
1	6	4	3
2	6	3	0
3	3	0	0
4	3	2	0
Average	4.5	2.3	0.8

Table 4.2. Least square means of mycelial growth of IL12-Ds-1 for six days on water agar plates of either 0%, 5%, 15%, or 25% of the As-1 culture filtrate. Least square means of mycelial growth followed by the same letter within a column are not significantly different using the Tukey-Kramer adjustment in multiple comparisons.

	Mycelial Growth (mm)					
Culture Filtrate (%)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
0	9 a	18 a	28 a	36 ab	49 a	56 a
5	8 a	18 a	28 a	40 a	50 a	59 ab
15	8 a	17 a	28 a	36 ab	45 ab	54 ab
25	9 a	18 a	25 a	32 b	41 b	49 b

Table 4.3. Least square means of percent germination of IL12-Ds-1 alpha-conidia incubated for 24 hours in the growth chamber in 0%, 25%, or 50% of the As-1 culture filtrate. Least square mean differences between the various concentrations of the culture filtrate listed in this table using the Tukey-Kramer adjustment were not significant.

Culture Filtrate (%)	Conidial Germination (%)
0	83
25	75
50	80

Table 4.4. Least square means of the percent rotted seed in the greenhouse experiment or the percent germinated seed in the growth chamber experiment inoculated with either IL12-Ds-1, As-1, or a combination of IL12-Ds-1 and As-1. Sterile double-distilled water was the control. Least square means followed by the same letter within a column are not significantly different using the Tukey-Kramer adjustment in multiple comparisons.

	Greenhouse	Growth Chamber
Treatment	Rotted Seed (%)	Seed Germination (%)
Control	27 ab	80 a
As-1	23 a	64 ab
IL12-Pl-1	60 b	28 c
IL12-Pl-1 + As-1	53 ab	38 bc

Discussion

The fungus that appeared to have parasitized the *D. sojae* isolate Li53 culture was identified as *A. strictum*, a known mycoparasite (Choi et al. 2008; Rivera-Varas et al. 2007), via morphological and phylogenetic analysis. Although the pycnidia-inhibiting sectoring that As-1 caused on the Li53 culture was not observed on *D. sojae* isolate IL12-Ds-1, *A. strictum* has been shown to greatly reduce conidial production of *Helminthosporium solani* Durieu & Mont. (Rivera-Varas et al. 2007). Unlike the *A. strictum* infecting *H. solani*, the As-1 isolate of *A. strictum* was not an obligate parasite of *D. sojae* as we were able to obtain a single-spore isolate of it. Additionally, while Rivera-Varas et al. (2007) reported that conidial germination of *H. solani* was reduced by *A. strictum* infection, our spore germination assay did not show any

significant difference in germination of alpha-conidia of IL12-Ds-1 incubated in various concentrations of the culture filtrate of As-1, and our leaf inoculation assay did not show any inhibition of pycnidial development by IL12-Ds-1. It is likely that As-1 released some kind of chemical compound that inhibited mycelial growth of IL12-Ds-1 based on the results of the culture filtrate dilution plate experiment and the dual culture experiment. Verlamelin, a possible candidate, has been shown to inhibit a variety of fungi in vitro (Kim et al. 2002). Interestingly, the methodology of Kim et al. (2002), which involved the use of filter paper discs to observe zones of inhibition, did not work as well as the dual culture experiment or the culture filtrate dilution plate experiment for obtaining an in vitro assessment of fungal inhibition. The late inhibition of mycelial growth of IL12-Ds-1 in the culture dilution plate experiment and the rapid growth of IL12-Ds-1 over the zone of inhibition of As-1 seem to suggest that As-1 does not produce large quantities of a mycelial-inhibiting compound sufficient to effectively inhibit IL12-Ds-1. It should be noted, however, that the control plates for the culture filtrate dilution plate assay did not contain any nutrients while the plates with some concentration of the culture filtrate did have nutrients, albeit reduced as As-1 used them up as it grew in the Erlenmeyer flask before filtration. The lack of inhibition of pycnidial development in the leaf assay and pre- and post-emergence damping off in the greenhouse and growth chamber seed assays might have been due to a combination of the poor mycelial inhibition abilities of As-1 and the protectant rather than a curative nature of *A. strictum* in its role as a biocontrol (Rivera-Varas et al. 2007; Choi et al 2009).

Given the protectant nature of *A. strictum*, biocontrol of PSD should be attempted by inoculating soybean plants before and during the seed development and maturity stages (R5-R8) (Fehr et al. 1971), which are the stages during which *D. sojae* infects soybean pods and seed, with *A.*

strictum conidia (Kmetz et al. 1978; Ploper et al. 1992; Tomes et al. 1985). *A. strictum* conidia have been found to protect plants better against pathogens than its culture filtrate (Choi et al. 2009). With the large quantity of conidia that *A. strictum* produces despite its slow mycelial growth, its potential value for the effective biocontrol of PSD should be investigated further. The fungus or compounds produced by it could be useful in maintaining the quality of soybean produced in the Mid-South, where planting of earlier-maturing cultivars has led to a decline in seed quality.

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